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14. ABSTRACT: The tumor suppressor p53 plays an important role in a variety of cancers including breast cancer. In response to oncogenic signals, it induces growth arrest, apoptosis or senescence. We are determining the role of p53 in human mammary epithelial cell (HMEC) senescence. Previously, we identified several transcriptional targets of p53 using array analysis In this reporting period, we confirmed expression of several of these targets by RT PCR. We also chose several other targets of p53 that are induced by DNA damage. The RT PCR analysis aws carried out using mRNA prepared from young growing early passage and senescent late passage HMECs. Results indicate that while several p53 target genes showed significant upregulation, certain other targets showed no significant upregaultion in senescent HMECs suggesting a differential regulation of p53 target genes during senescence in HMECs. We are further studying the regulation of these genes by p53 using chromatin immunoprecipitation linked PCR (ChIP). We are also determining the role of these genes in HMECs senescence by RNA interference approach. IGFBP3, TGFα, Wig1, HGFL, BCL6, TMEM30A, BTG-2, GADD45A and MDM4 showed the most significant upregulation in senescent HMECs. To identify additional targets of p53 that are induced during HMEC senescence, we are making a ChIP library of p53 target genes using antibodies that are specific to total and phospho-p53, and cells that are undergoing cellular senescence.

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Table of Contents

INTRODUCTION

Breast cancer is a complex multistep process involving several molecular genetic changes (1). In vitro studies in culture suggest that first molecular genetic change entails bypass of cellular senescence followed by the immortalization of cells (1, 2). These changes allow cells to accumulate further mutations, which results in a transformed phenotype (1). It is believed that breast cancer arise due to transformation of human mammary epithelial cells (HMECs), which line the ducts of mammary gland (1)

After completing a certain number of divisions, normal cells enter a state of irreversible growth arrest and altered function, known as cellular senescence (3), which is considered a tumor suppressive mechanism (3- 5). In somatic cells, telomerase remains repressed and telomere length keeps shortening at each round of DNA replication. Short telomeres signal cells to stop further proliferation and invoke cellular senescence (6, 7). Two important tumor suppressor pRb and p53 are required for the maintenance and genesis of senescent phenotype (3-5)

The p53 protein is a typical transcription factor and contains an N-terminus transactivation, a centrally located DNA binding and a C-terminus oligomerization domains (8). Transcriptionally active p53 binds to a consensus site 5' -RRRCA/TA/TYYY-3', often present in pairs in p53 regulated genes (8). Tumor derived mutants of p53 are always defective in sequence-specific transactivation, thus attesting the importance of transcription activation function of p53. In response to various physiological stimuli, p53 undergoes post-translational modifications such as phosphorylation and acetylation, which activate p53 transcription functions (8, 9). Activation of these transcription activation functions results in either apoptosis, G1 and G2 cell cycle arrest or senescence (8-10).

When mammary tissue is explanted in an appropriate tissue culture medium, a heterogeneous cell population emerges (11). This heterogeneous population proliferates for 3-5 population doublings before a majority of cells undergoes senescence. Regular feeding of these cells (sometimes) give rise to a homogeneous population which is referred to post-selection HMECs, while the original heterogeneous mixture is referred to as pre-selection cells (11, 12). Senescence in pre-selection cells, which is also termed as M0 stage, appears to be due to the accumulation of p16 (13), and emergence of post-selection homogeneous culture is believed to arise due to progressive methylation of p16 locus (11-14). However, post-selection cells still undergo senescence and never spontaneously immortalize. Since in post-selection HMECs, p16 is methylated, it is thought that pRb-p16 pathway does not play a role in senescence in these cells. Hence, we are interested in exploring the role of p53-p21 pathway in senescence in post-selection HMECs.

In the previous progress reports, we showed that p53 and p21 is significantly upregulated during senescence in post-selection HMECs but not in pre-selection HMECs. In post-selection HMECs, using RNAi approach, we also showed that p53 knockdown was much more effective than p21 knockdown in overcoming senescence and extending the replicative life-span of HMECs, suggesting the role of additional p53 target genes in HMEC senescence. Recently, using a p53 target gene array we identified additional p53 target genes that are upregulated in posr-selection HMECs. Here, we confirmed and studied the role of these additional target genes in HMEC senescence. We also selected few recently described p53 target genes (15-17) and found the differential upregulation of p53 target genes during senescence in HMECs.

BODY:

Post-selection 76N and 76NhTERT (telomerase immortalized 76N cells) were obtained from our collaborator Dr. Vimla Band. These cells were cultured in DFCI-1 medium as described (19). Cells were serially passaged in culture until senescence. Senescence was determined using senescence associated beta-galactosidase (SA-β-gal) assay and using ^{3}H-thymdine incorporation assay (% labeled nuclei or %LN) as described (20,21). Cells were considered early passage when >70% cells incorporated ^{3}H-thymidine and less than 5% cells were SA-β-gal positive. Conversely, cells were considered late passage or senescent when SA-β-gal index was >70% and %LN were 10-15%. SA-β-gal is a widely used senescence marker used in various cell types including HMECs. Total RNA was prepared from early (growing) and late (senescent) HMECs and RT PCR analysis was done as described (22). The primer sequences and expected product sizes are given in following table.

Table 1

Gene	Forward Primer	Reverse Primer	Product size (bp)	Reference #
DDB2	TTACTCTGCTTCCCAGTG	GCTCCAGATGAGAATGTG	**298 bp**	**15**
UBTD1	CCATCTACTGCCTGTCAC	GATGATGACCTGGATGAC	**293 bp**	**15**
TMEM30A	GGTACAACAAAGCCTGTG	CAGCGATGTAAGCAATCC	**292 bp**	**15**
RPS27L	TACATCCGTCCTTGGAAG	TGAACACCCTTCTGTGAG	**194 bp**	**15**
TP53AP1	GCCTGACCCAGGATCTAG	CACTGGTGTAAGTGTTCG	**183 bp**	**16**
MDM4	GGCTCCTGTCGTTAGAC	CCCCAGCCTTCTTTAGTC	**296 bp**	**16**
ANKRD2	GAGGGATAAGCTGCTGAG	CAGCCCGTTATGCTCAG	**298 bp**	**15**
GADD45A	ATCACTGTCGGGGTGTAC	CTTAAGGCAGGATCCTTC	**299 bp**	**16, 17**
BTG2	CAAACACCACTGGTTTCC	ACTGCCATCACGTAGTTC	**300 bp**	**16, 17**
ANXA4	ACCGAAATCACCTGTTGC	AGTCTCCAGATGTGTCAC	**294 bp**	**17**
IGF-BP3	TAAAGACAGCCAGCGCTA	CTGCCCATACTTATCCACA	**252**	**16, 18**
TGF-α	TCAGTTCTGCTTCCATGG	TTTCTGAGTGGCAGCAAG	**299 bp**	**16, 18**
Wig1	CGGAAGCTCAGAGTAACTC	CTCCATCTCATTCCTGTACC	**300 bp**	**16, 18**
HGFL	GAAGGAGCAGTGGATACTGAC	GGACTGTGTCATTACCCGTAC	**299 bp**	**16, 18**
BCL6	CAGTGACAAACCCTACAAG	GCTCTTCAGAGTCTGAAGG	**300 bp**	**16, 18**

RGS 14	GTGTGAAGATCTCCAAAGC	CTGCTGATTTGGTCTGTG	**297 bp**	**16, 18**
PPM1D	CTCGAGAGAATGTCCAAGG	GCTGAGCACCACTACTTC	**300 bp**	**16, 18**
TSP-1	GGACAACTGTCCCTATGTG	CCAGTTAGGGTCATTTTGG	**300 bp**	**16, 18**

The RT PCR data (Fig 1) suggest that IGBP3, TGFα, Wig1, HGFL, BCL6, TMEM30A, BTG-2, GADD45A, MDM4 are highly expressed in senescent HMECs.

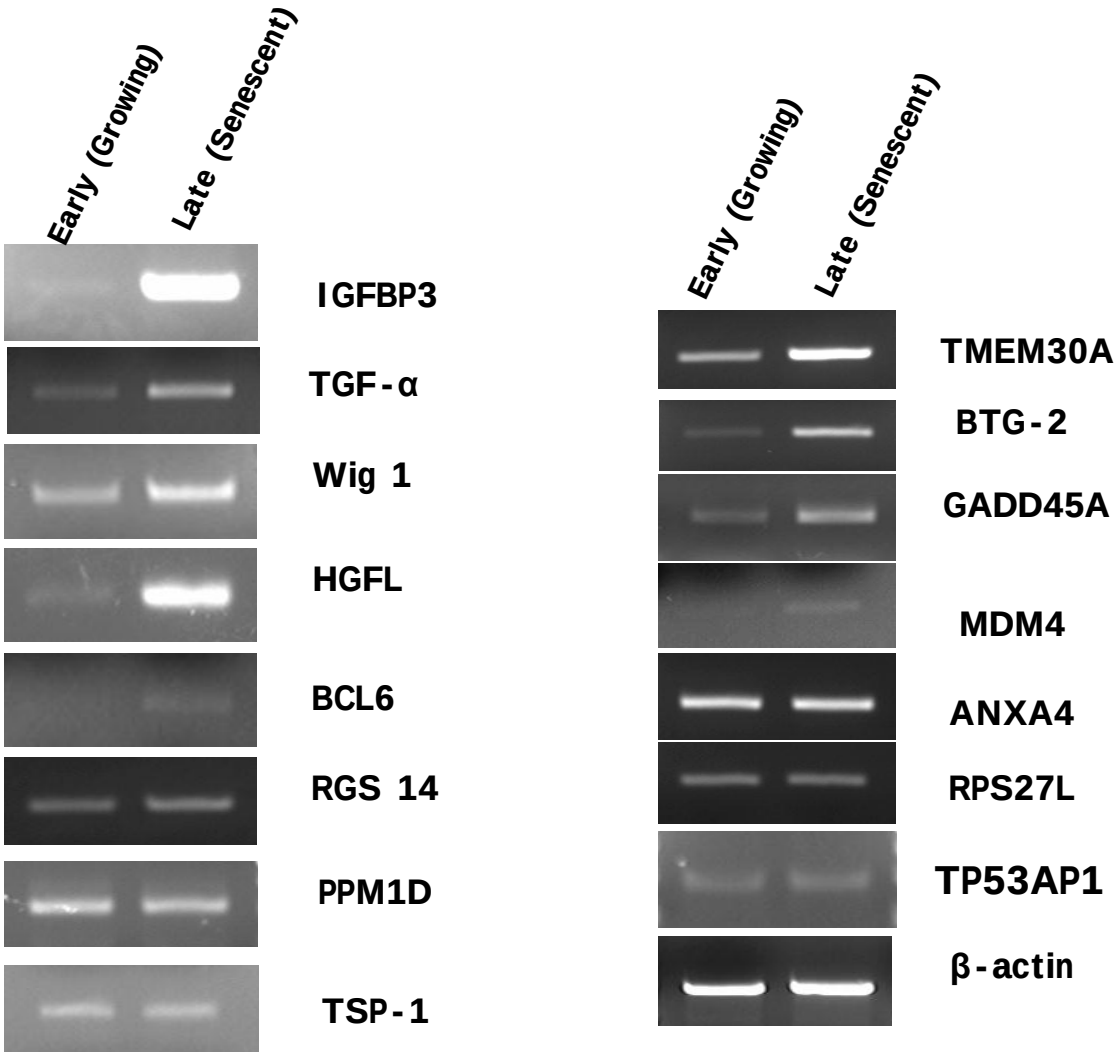

Figure 1: RT PCR analysis of selected p53 target genes in growing and senescent HMECs. ß-Actin was used as a loading control.

Next, we are optimizing ChIP assay to clone senescence specific targets of p53 in HMECs. ChIP assay was done using chromatin Immunoprecipitation kit from Upstate Cell Signaling Solutions (Charlottesville, Virgina) as described by manufacturer. Briefly, chromatin

DNA was cross-linked with proteins/transcription factors (e.g. p53) in cells using 1% final formaldehyde treatment of cells for 10 min at room temperature. The cells were washed with cold phosphate buffered saline containing protease inhibitors and scrapped in PBS and pelleted by centrifugation. The cell pellet was lysed in SDS lysis buffer and chromatin was sonicated to shear DNA length of ~.5 Kb. ChIP dilution buffer was added to sonicated DNA and protein A-agarose-Salmon-sperm DNA slurry was added to supernatant, and the mixture was incubated for 30 min. After incubation and a brief centrifugation, the supernatant was collected and 10 ug of p53 antibody (DO1, Santa Cruz Biotech, Santa Cruz, CA) was added to it. After overnight incubation, the p53-IPed chromatin was collected by incubating with Protein A agarose beads and brief centrifugation. The beads were washed serially with low salt and high salt immune complex wash buffers (2X each). The DNA-protein complexes were reverse cross linked and proteins were digested by proteinase K treatment.

The DNA was precipitated and PCR was performed for selected p53 target genes using primers flanking p53 binding sites. Immunoprecipitation using mouse IgG was used as a negative control. 10% of initial lysed and sonicated cell extract was used as input for PCR after phenol extraction and DNA precipitation. Initially, we compared ChIP assay pattern of growing and senescent cells for p53 target genes p21 and PIG3. The primer sequence is given in Table 2. The results (Fig. 2) indicated that p53 was indeed highly bound to p21 promoter, although no enhanced binding was detected to PIG3 promoter. Next, the mixture of DNA after chromatin IP from senescent cell was treated with klenow (DNA polymerase) and cloned as blunt end fragments in a positive selection cloning vector pZErO (Invitrogen). Our initial attempt of cloning resulted in very few clones indicating that the IPed DNA quantity was not sufficient to result in high efficiency cloning. We are currently working on to scale up the ChIP assay using more senescent cells to isolate substantial quantity of p53 ChIPed DNA. Otherwise, the p53 ChIPed DNA from several experiments will be pooled and cloned in pZErO vector. Several hundred resulting clones will be sequenced to identify novel p53 target genes.

Table 2

Gene	**Forward Primer**	**Reverse Primer**	**Product size (bp)**
P21	GTGGCTCTGATTGGCTTTCT	CTGAAAACAGGCAGCCCAAG	100 bp
PIG-3	GCCCATCTTGAGCATGGGT	CACTCCCAACGGCTCCTTT	94 bp
GAPDH	GTATTCCCCCAGGTTACAT	TTCTGTCTTCCACTCACTCC	100 bp

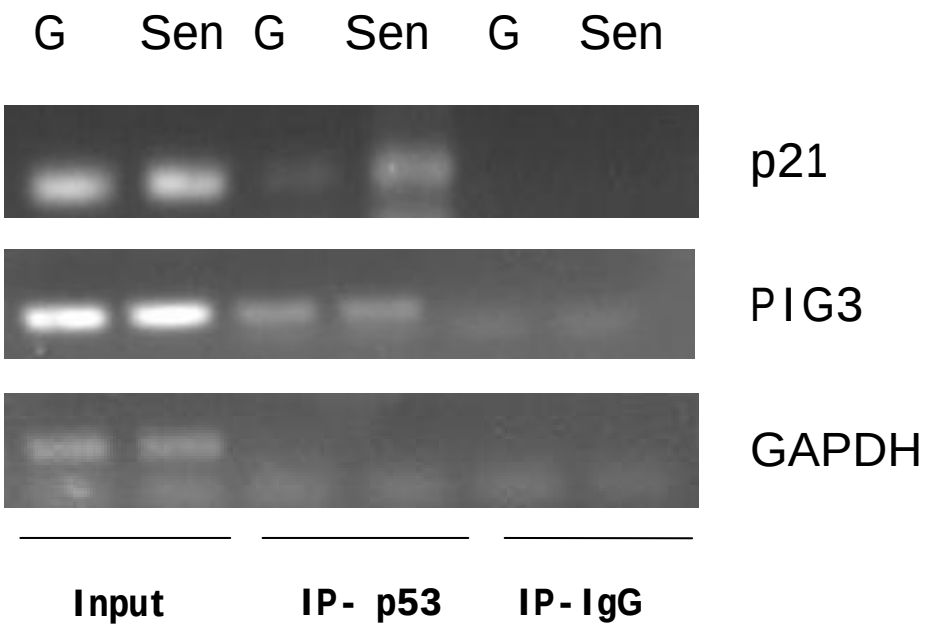

Figure 2: ChIP analysis of p53 targets in growing (G) and senescent (Sen) cells. ChIP assay was performed as described in text. GAPDH is a negative control for ChIP PCR. IP-Ig is a negative control for ChIP assay.

KEY RESEARCH AND TRAINING ACCOMPLISHMENTS:

- During past four years, I have learned how to culture pre- and post-selection HMECs, and determine senescence in these cells.
- We have developed HMECs cell lines stable expressing p53 and p21 RNAi and mdm2. We will use these cells for further transformation studies and generating cell culture model of breast cancer.
- We have analyzed p53 arrays for the expression of p53 target genes. Our data suggest role of additional p53 target genes in HMEC senescence.
- We have confirmed upregulation of several of p53 target genes by RT PCR.
- We have also optimized p53 DNA binding and chromatin-immunoprecipitate linked PCR (ChIP) assay.
- This training support has also helped me developed my research program related to breast cancer, particularly studying the role of various regulators of senescence such as polycomb proteins. A manuscript related to role of polycomb proteins in breast cancer is currently under review (copy of submitted manuscript included in appendix).
- This training support has helped me to develop my professional career and as a result, I am now recognized as a key researcher in the field of breast cancer and senescence as evidenced by my CV, which is enclosed in appendix.

REPORTABLE OUTCOMES: Following publications/abstracts were supported by this training grant.

Publications:

1. **Dimri, G.P.** (2005) What has senescence got to do with cancer? **Cancer Cell** 7: 505-512.

2. **Dimri, G. P.**, Band, H and V. Band (2005) Mammary epithelial cell transformation: insights from cell culture and mouse models. **Breast Cancer Res.** 7: 171-179.

Abstract:

1. Yadav, A., Datta, S., Band, V. and **Dimri, G. P.** (2005) Role of p53 in Mammary Epithelial Cell Senescence. Era of Hope, Dept. of Defense, Breast Cancer Research Program Meeting. Abst # P27-8.

CONCLUSIONS:

p53 an important mediator of cellular senescence, which plays a role in telomere length dependent senescence. Gradual telomere shortening is thought to provoke a DNA damage checkpoint mediated by p53, which results in permanent growth arrest. Most tumor cells have lost this ability to undergo senescence and cycle even when telomere lengths critically short. In this report, we have presented evidence that p53 may play an important role in senescence of post-selection cells but not pre-selection cells.

In the first year of the grant, we proposed to study the DNA binding activity, its expression level and posttranslational modifications during senescence in HMECs. We have completed the proposed studies. However, we have not found any significant differences in posttranslational modifications using limited number of antibodies that we used.

In the second year, we started using p53 RNAi approach to study the role of p53 in senescence. We generated post-selection HMECs cells with p53 and p21 knockdown using RNAi approach. The study of replicative life span of these cells suggest that p53 plays an important role in senescence of post-selection HMECs and other target genes of p53 are possibly involved in senescence. Next year we plan to perform p53 ChIP analysis in post-selection HMECs and identify additional targets of p53 involved in HMEC senescence as previously proposed in the grant application.

In the third year we have generated mdm2 overexpressing HMECs. Cells expressing p53 and/or p21 RNAi, and mdm2 are further being characterized in terms of senescence. In third year, we have also carried out p53 array analysis to examine the expression of p53 target genes during senescence. We have selected few p53 target genes for further study. These genes are likely to play a role in breast cancer.

In the fourth year, we have confirmed the upregulation of several additional p53 target genes by RT PCR analysis. We have made progress in making a ChIP library of p53 target genes in senescent cells to identify novel targets of p53 that may play a role in HMEC senescence and breast cancer.

Since, two and half years ago, I moved to my new host institute and transfer of this training grant took quite some time, completion of experiments outlined in the original proposal got delayed. Due to delay in grant transfer and setting up of the new lab, the grant is under no cost extension period. During this period, we are continuing our research to develop ChIP library and study the function of additional target genes of p53 in senescence of mammary epithelial cells and breast cancer.

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Review

Mammary epithelial cell transformation: insights from cell culture and mouse models

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Abstract

Normal human mammary epithelial cells (HMECs) have a finite life span and do not undergo spontaneous immortalization in culture. Critical to oncogenic transformation is the ability of cells to overcome the senescence checkpoints that define their replicative life span and to multiply indefinitely – a phenomenon referred to as immortalization. HMECs can be immortalized by exposing them to chemicals or radiation, or by causing them to overexpress certain cellular genes or viral oncogenes. However, the most efficient and reproducible model of HMEC immortalization remains expression of high-risk human papillomavirus (HPV) oncogenes E6 and E7. Cell culture models have defined the role of tumor suppressor proteins (pRb and p53), inhibitors of cyclin-dependent kinases ($p16^{INK4a}$, p21, p27 and p57), $p14^{ARF}$, telomerase, and small G proteins Rap, Rho and Ras in immortalization and transformation of HMECs. These cell culture models have also provided evidence that multiple epithelial cell subtypes with distinct patterns of susceptibility to oncogenesis exist in the normal mammary tissue. Coupled with information from distinct molecular portraits of primary breast cancers, these findings suggest that various subtypes of mammary cells may be precursors of different subtypes of breast cancers. Full oncogenic transformation of HMECs in culture requires the expression of multiple gene products, such as SV40 large T and small t, hTERT (catalytic subunit of human telomerase), Raf, phosphatidylinositol 3-kinase, and Ral-GEFs (Ral guanine nucleotide exchange factors). However, when implanted into nude mice these transformed cells typically produce poorly differentiated carcinomas and not adenocarcinomas. On the other hand, transgenic mouse models using ErbB2/neu, Ras, Myc, SV40 T or polyomavirus T develop adenocarcinomas, raising the possibility that the parental normal cell subtype may determine the pathological type of breast tumors. Availability of three-dimensional and mammosphere models has led to the identification of putative stem cells, but more studies are needed to define their biologic role and potential as precursor cells for distinct breast cancers. The combined use of transformation strategies in cell culture and mouse models together with molecular definition of human breast cancer subtypes should help to elucidate the nature of breast cancer diversity and to develop individualized therapies.

Introduction

More than 80% of adult human cancers are carcinomas, tumors originating from malignant transformation of epithelial cells. However, much of our understanding of oncogenic transformation comes from fibroblast transformation systems. Breast cancer is the second leading cause of cancer-related deaths among women in the USA [1]. The vast majority of breast cancers are carcinomas that originate from cells lining the milk-forming ducts of the mammary gland (for review [2]). Deliberate transformation of these cells provides a practical window into human epithelial oncogenesis. Malignant transformation represents a complex multistep process in which genetic, environmental, and dietary factors together are thought to alter critical cell growth regulatory pathways resulting in uncontrolled proliferation, which is a hallmark of tumorigenesis [3,4]. Understanding the nature of these cellular pathways is of central importance in cancer biology.

The growth of normal human mammary epithelial cells (HMECs), which include luminal, myoepithelial and/or basal cells (described below), is tightly controlled. These cells grow for a finite life span and eventually senesce (for review [5-7]). Both cell culture and mouse models have provided evidence that essential initial steps in tumorigenesis involve the loss of senescence checkpoints and immortalization, which allow a cell to grow indefinitely and to go through further oncogenic steps, resulting in fully malignant behavior. In addition, cell culture model systems have identified a number of genes whose alterations are involved in HMEC immortalization and thereby have provided significant insights into the biology of early breast cancer [5,7,8]. Use of oncogene combinations has allowed researchers to create cell culture models of full HMEC transformation, thereby illuminating the process of

ASMA = α-smooth muscle actin; CDK = cyclin-dependent kinase; COX = cyclo-oxygenase; ER = estrogen receptor; ESA = epithelial-specific antigen; HMEC = human mammary epithelial cell; HPV = human papillomavirus; hTERT = catalytic subunit of human telomerase; PD = population doubling; Ral-GEF = Ral guanine nucleotide exchange factor; TDLU = terminal ductal–lobular unit.

breast cancer progression [9-11]. Additional insights have come from mouse models, using transgenic overexpression of oncogenesis-promoting genes and deletion of tumor suppressor genes, which often produce breast adenocarcinomas that closely resemble human breast cancers.

Studies using cell culture transformation models have pointed to the existence of HMEC subtypes with distinct susceptibilities to oncogenesis by different oncogenes [5,8]. Remarkably, direct cDNA microarray profiling of human breast cancers has led to similar insights, identifying multiple subtypes of human breast cancer with distinct outcomes; phenotypic and genotypic characteristics of these breast cancer subtypes point to their possible origin from specific subtypes of HMECs, such as basal or luminal cells [12]. Finally, cell culture and mouse model systems have begun to identify mammary stem cells that may provide progenitors for oncogenic transformation [13] and have led to an appreciation of the microenvironment for oncogenesis [14,15].

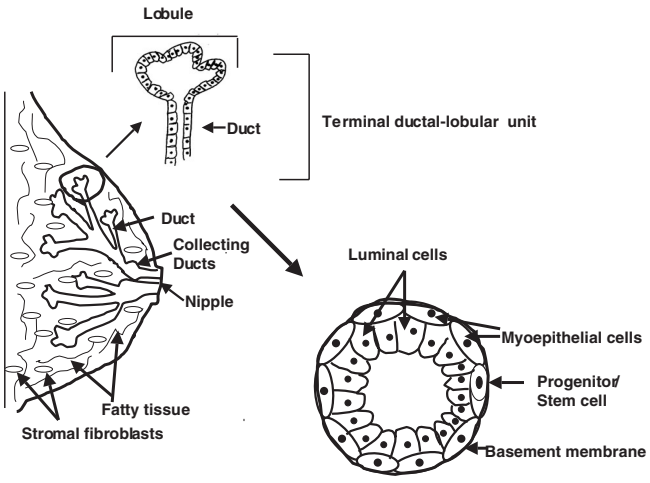
Thus, studies conducted over the past several years have established the importance of HMEC transformation models to our understanding of the pathways that control normal mammary cell growth, development, and oncogenesis. However, many challenges remain, including the identification of mammary cell subtypes or oncogenic strategies that result in cancers that resemble naturally occurring human breast cancers, and translation of new research to devise more specific diagnostic and treatment strategies for different subtypes of breast cancer.

Mammary gland and various epithelial cell subtypes

The mammary gland consists of a branching ductal system that ends in terminal ducts with their associated acinar structures, termed the terminal ductal–lobular units (TDLUs), together with interlobular fat and fibrous tissue [16,17]. Most breast cancers arise in the TDLU (Fig. 1). Unlike other epithelial cancers, such as that of colon, different stages of breast cancer are not clearly defined. However, it is clear that benign stages (such as typical and atypical hyperplasia), noninvasive cancers (such as carcinoma *in situ* – ductal or lobular), and invasive cancers (such as invasive ductal or lobular carcinomas) do exist. Additionally, multiple types of *in situ* carcinomas, such as solid, cribiform, papillary and comedo types, have been reported and it is possible that these represent tumors originating from different epithelial subtype [16,17].

Histological examination of TDLU reveals two major types of cells: inner secretory luminal cells and outer contractile myoepithelial cells (Fig. 1). In addition to luminal and myoepithelial cells, there is emerging evidence that basal cells (presumed to be the progenitor for myoepithelial cells) and stem cells exist in the TDLU [17,18]. Until recently it was believed that the vast majority of breast carcinomas arise from luminal epithelial cells [2]. This was based on the keratin expression and other phenotypic markers of cultured tumor cell lines, mostly derived from metastatic lesions [2]. Unfortunately, the great majority of primary breast tumors have proved difficult to establish in cultures, either on plastic or as three-dimensional cultures [5-7,19-21]. However, recent molecular profiling studies clearly show the existence of multiple subtypes of breast cancers probably originating from luminal, basal, and possibly stem cell compartments [12] (described below in detail).

Figure 1



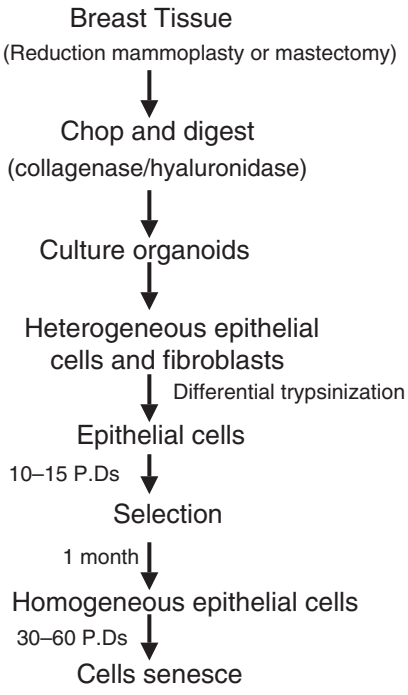
Structure of the mammary gland. Terminal ductal–lobular unit (TDLU), composed of ductal cells, is the unit thought to be the origin of most breast cancer. The stroma is composed of fatty tissue (adipocytes) and fibroblasts. Also shown are the two primary types of cells in normal ducts: outer contractile myoepithelial and inner columnar luminal cells. A putative progenitor/stem cell is also indicated.

Culturing of various epithelial cell subtypes

For more than two decades, various investigators have attempted to develop cell culture models that lead to isolation of breast cancer cells resembling those found in human breast cancers. In order to establish such models, it was essential to culture normal HMECs. In 1980s, work from several laboratories showed that normal HMECs could be cultured in cell culture [22,23] (for review [2,5,7]).

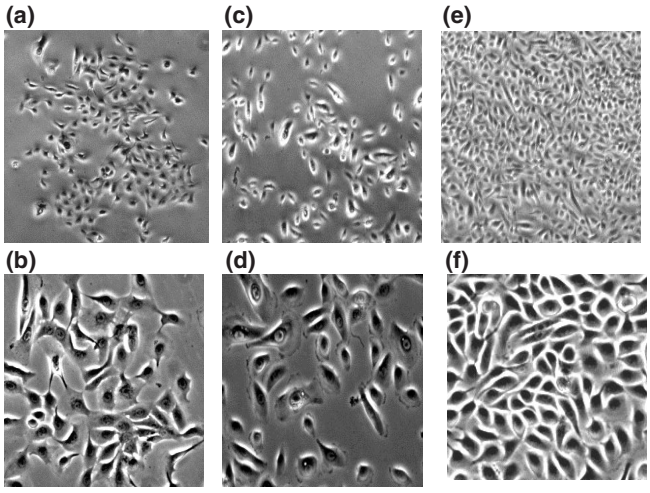
In our laboratory we defined a medium, termed DFCI-1, that helped us to establish and culture normal and some primary breast cancers under identical conditions [20]. However, in general the difficulty in establishing primary tumor cells in cell culture has persisted. Notably, early cultures derived from reduction mammoplasty or mastectomy specimens exhibit considerable heterogeneity (with multiple cell types – luminal, stem cells, basal and myoepithelial cells) and grow for three to four passages or about 15–20 population doublings (PDs), and then senesce (Figs 2 and 3) [5-7]. The senescence in these cells is also termed as M0 stage [24].

Figure 2



Establishment of mammary epithelial cells from reduction mammoplasty/mastectomy specimens. The tissue is chopped, digested with collagenase and hyaluronidase, and plated in medium as organoids. Over a week or so, multiple types of epithelial cells and fibroblasts emerge; fibroblasts are removed by differential trypsinization (fibroblasts are loosely attached), remaining epithelial cells grow for 10–15 population doublings (PDs) followed by senescence of the majority of cells. Occasionally, an homogenous population of cells emerges that continue to proliferate for an additional 30–60 PDs, and eventually these cells also senesce (this step is referred to as agonescence).

Figure 3



Morphological heterogeneity of cells before and after selection. **(a–d)** Two views of mammary epithelial preselection cells (original magnifications: panels a and c, 40×; panels b and d, 100×). Cells shown in panel a grow as compact clusters and are relatively uniform, whereas cells in panel b grow more dispersed and exhibit different types of cells (small and large). **(e,f)** Views of postselection human mammary epithelial cells with relatively uniform morphology (original magnifications: panel e, 40×; panel f, 100×).

However, in some cases (not always) an occasional homogenous cell population emerges that continue to grow further for 30-60 PDs (Figs 2 and 3) [5-7] before senescence occurs (also called agonescence, described below) [25]. This process of emergence of cells that are able to proliferate for extended periods is also known as self-selection; before selection the cells are termed preselection cells, whereas those that emerge after selection are called postselection cells. The keratin profile of preselection cells (K-5, K-6, K-7, K-14, K-17, K-18 and K-19 positive) [8,19,26] suggests the existence of both luminal and basal (myoepithelial) cells. However, postselection cells generally exhibit a loss of expression of K-19 but retain the expression of all other keratins [8,18,25]. These cells also express α-smooth muscle actin (ASMA), suggesting that these may be of myoepithelial origin. Further development of cell sorting techniques and chemically defined media have helped in culturing of luminal and progenitor epithelial cells [14,27] (described below in detail).

It has also been reported that postselection cells lose the expression of $p16^{INK4a}$, a cyclin-dependent kinase (CDK) inhibitor [24,25], and gain expression of cyclo-oxygenase (COX)-2, a gene that is thought to be involved in tumorigenesis [28]. As both of these genes are implicated in oncogenesis, it is conceivable that loss of p16 or gain of COX-2 expression may make these cells more susceptible to transformation, although it is unclear whether the loss of p16 and gain of COX-2 occur *de novo* during self-selection or represent selection of a minor population of cells with pre-existing high COX-2 and low p16 expression. Notably, p16-negative and COX-2-positive cells could be detected using immunohistochemistry in normal mammary tissue [28,29].

Immortalization of various HMEC subtypes in culture

As alluded to above, normal mammoplasty-derived HMECs exhibit a limited life span, which is followed by replicative senescence. Replicative senescence acts as a strong tumor suppressor mechanism and prevents spontaneous immortalization of human cells [30-33]. A major determinant of replicative senescence is the enzyme telomerase, which maintains the length of telomere ends [30,31]. Most somatic cells express little or no telomerase, resulting in telomere shortening with successive cell divisions, which eventually elicits a senescence checkpoint [30-32]. A senescence-like phenotype can also be induced by a variety of nontelomeric signals such as DNA-damaging agents, adverse cell culture conditions, and overexpression of certain oncogenes [30,32]. The tumor suppressor protein p53 and its target gene product p21, and $p16^{INK4a}$ play a crucial role in senescence

induced by telomeric as well as nontelomeric signals [30-33]. Much of our knowledge about senescence comes from studies conducted in human fibroblasts [30-34]. Only recently have we begun to elucidate the mechanisms of senescence in epithelial cells, in particular in HMECs [25].

The senescence associated with the 'selection' phase in HMEC cultures is accompanied by classic features of senescence, such as flat morphology, presence of vacuoles, and positive staining for senescence-associated β-galactosidase (SA-β-gal), a marker of senescence [34]. The block in cell proliferation at this stage is dependent on the pRb/p16 pathway [24,35], because the human papillomavirus (HPV) oncogene E7, which binds and inactivates pRb, can overcome the M0/selection stage [36]. Similarly, a constitutively active p16-insensitive CDK4 mutant can overcome the M0 stage [37]. Thus, senescence of preselection cells appears to be telomere independent. At the end of their replicative life span, postselection HMECs exhibit senescence as well as cell death with a high level of genomic instability. This phenomenon is termed as agonescence, as opposed to replicative senescence [25]. Most importantly, unlike rodent cells, human HMECs derived from reduction mammoplasties or from milk do not exhibit spontaneous immortalization and thus provide suitable models of human cell transformation. Immortalization of HMECs in culture is characterized by their continuous growth beyond the agonescence checkpoint. It is thought that immortalization is an early step in human cancer, and continued proliferation of immortal cells allows the accumulation of additional genetic changes that promote malignant and metastatic behavior.

Stampfer and Bartley [38] presented initial evidence that HMECs could be immortalized in cell culture using benzo(a)-pyrene; however, the immortalization was a rare event in this case. Similar to carcinogen-induced immortalization, we found that γ-radiation induced the transformation of HMECs relatively infrequently [5,8,39]. In general, most viral oncogenes (including SV40 T antigen, adenovirus E1A and E1B, polyoma T antigen) have not proven very efficient as immortalizing genes for human cells [40]. While the introduction of the SV40 T antigen into breast tumor tissue-derived epithelial cells gave rise to immortal cell lines, SV40-transfected cells go through a long crisis period, and emergence of immortal cells is rare [19]. Over the past several years, our studies have defined a system to immortalize human HMECs efficiently and reproducibly, using the urogenital carcinoma-associated HPV oncogenes E6 and E7 [5,8,36].

Comparison of early (preselection) and late-passage (post-selection) cultures revealed that different HMEC subtypes exhibit a remarkably distinct susceptibility to E6 or E7, or their combination [8]. One HMEC subtype was exclusively immortalized by E6 but not by E7; such cells predominated the late-passage cultures but were rare at early passages. Surprisingly, a second cell type, present only in early passages of tissue-derived cultures, showed extension of life span and infrequent immortalization by E7 alone. Finally, E6 and E7 together were required to immortalize fully a large proportion of preselection HMECs [8].

Human milk is an easily available source of relatively pure HMECs that are thought to be differentiated luminal cells [2,19]. However, these cells can be cultured for only a limited number of passages (typically two to three passages, or five to nine PDs), which has precluded their detailed biochemical study [2,18]. Most of the work on milk cells has been carried out in Taylor-Papadimitriou's laboratory and has demonstrated that these cells can be immortalized by SV40 T antigen [41]. Interestingly, neither E6 nor E7 alone could induce the immortalization of milk-derived HMECs, whereas a combination of E6 and E7 was effective [8].

The reproducibility and relatively high efficiency with which E6 (in postselection HMECs) or E6 and E7 combined can induce immortalization of human HMECs have therefore yielded a practical approach to elucidate the biochemical mechanisms of HMEC immortalization. In recent years, using Yeast Two-hybrid analysis, we identified several novel targets of the E6 oncogene in HMECs. These targets represent novel mediator of HMEC immortalization [5]. These include ADA3 (alteration/deficiency in activation 3), a novel coactivator of p53 and steroid receptors (estrogen receptor [ER] and retinoic acid receptor) [42-44]; E6 targeted protein 1 (E6TP1), a novel GTPase activating Rap small G protein; and protein kinase N (PKN), an effector for Rho small G protein [5]. We recently found that MamL1, a human homolog of the *Drosophila* mastermind gene and a known coactivator for Notch [45], also interacts with E6 (I Bhat, V Band, unpublished data). These studies have implicated the p53, Notch, ER, Rho, and Rap signaling pathways in early transformation of human HMECs. Consistent with these analyses, we have shown that expression of mutant p53 [46] or activated Rho (X Zhao, V Band, unpublished data) induces immortalization of HMECs. Furthermore, several studies support a role for p53 mutations as an early event in breast cancer [47]. Taken together, these studies demonstrate that E6 is the most efficient immortalizing gene for postselection HMECs and that E6 immortalizes the HMECs by concurrently altering multiple biochemical pathways. Future studies will need to address the precise role played by these novel oncogene targets in early breast cancer.

In addition to viral oncogenes, alterations in the expression of cellular genes can also help to overcome senescence and promote HMEC immortalization. Among the cellular genes, we recently reported that Bmi-1, a member of the polycomb group of transcriptional repressors, could immortalize postselection HMECs [48]. Although the detailed mechanism of immortalization induced by Bmi-1 remains to be explored, Bmi-1 does not appear to immortalize these cells by down-regulating the INK4a/ARF locus. Interestingly, recent studies have implicated Bmi-1 in stem cell function and renewal

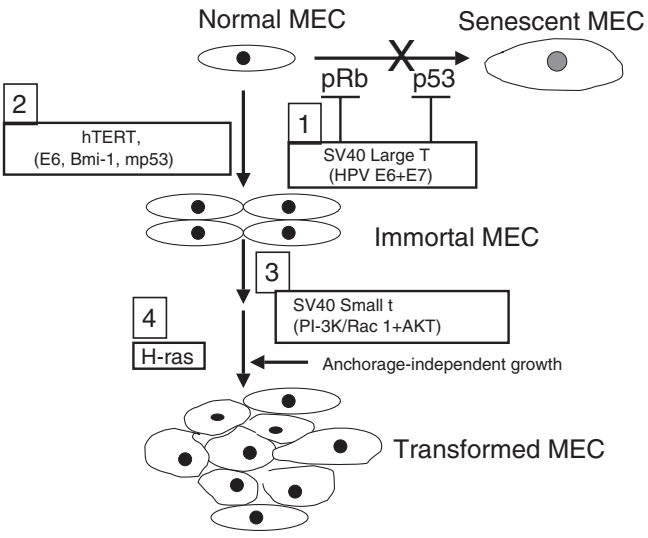
[49,50], suggesting that Bmi-1 could function as a potential breast cancer stem cell marker [50]. Another study showed that ZNF217, a zinc finger protein that is overexpressed in breast cancers, can promote immortalization of postselection HMECs [51]. Furthermore, introduction of hTERT also induces immortalization of postselection cells [5]. Interestingly, induction of telomerase has been documented early after E6 was introduced into HMECs [52], although the cause and effect relationship between telomerase induction and E6-induced immortalization continues to be debated. Recently, the E6 and E6–AP binding protein NFX-91 was implicated in E6-mediated induction of telomerase [53].

Cell culture models of full transformation of HMECs

The ability of researchers to establish normal HMECs and to induce their reproducible immortalization has provided momentum for further efforts to define the nature of biochemical alterations that can lead to full oncogenic transformation. As we and others have demonstrated, HMECs immortalized by most currently known procedures (such as E6 or E6 plus E7, mutant p53, Bmi-1 and hTERT) are preneoplastic and do not grow in an anchorage-independent manner or produce tumors when implanted in immune-deficient mice [5,8]. Weinberg and colleagues [9] recently established a multistep model of full HMEC transformation in cell culture by serial introduction of SV40 large T and small t, hTERT, and activated Ras (Fig. 4). It was shown that introduction of the SV40 large T, which binds and inactivates p53 and pRb, abolished senescence, whereas hTERT was needed to promote immortalization [9]. Notably, these studies showed an essential role for the SV40 small t, which inhibits protein phosphate 2A [54]. HMECs transformed by this method exhibited anchorage independence and produced poorly differentiated carcinoma (but not adenocarcinoma) when implanted in nude mice [9]. Further dissection of the role of small t revealed the importance of the downstream targets of phosphatidylinositol 3-kinase, Akt1 and Rac1, and direct activation of these pathways could fully substitute for small t in the transformation assays [10]. A recent refinement of the transformation in cell culture scheme suggests that perturbation of p53, pRb, protein phosphate 2A, telomerase, Raf, and Ral guanine nucleotide exchange factor (Ral-GEF) pathways are required for the full tumorigenic conversion of normal human cells [11]. The requirement in terms of modulating Raf and Ral-GEF pathways is cell type specific; HMECs require activation of Raf, phosphatidylinositol 3-kinase and Ral-GEFs, whereas human fibroblasts require the activation of Raf and Ral-GEFs [11]. Thus, serial use of viral and/or cellular genes is beginning to unravel the various combinations of genetic lesions that can convert a completely normal mammary epithelial cell into a fully tumorigenic one.

Although these studies have thus far relied on the use of known oncogenes, future studies using the cell culture transformation models with gene libraries should help identify novel cellular genes that participate at various steps of breast cancer progression. Vast majority of human breast cancers are adenocarcinomas, and only a small portion of breast cancers are poorly differentiated carcinomas. Hence, it appears that HMEC transformation in culture system is not optimal because the tumors produced by these transformed HMECs have usually been poorly differentiated carcinomas rather than adenocarcinomas. Breast cancer is associated with overexpression of various cellular proto-oncogenes such as ErbB2, epidermal growth factor receptor, Src family kinases, Bmi-1, cyclin D_1, cyclin E, CDK4, and other potential growth regulators. Use of these oncogenes in the multistep model described above and the use of other HMEC subtypes (such as luminal cells, potential stem cells, or those derived from milk) as a starting population may help to achieve full transformation of HMECs that develop into adenocarcinomas

Figure 4



Current consensus: normal HMECs can be fully transformed in definable serial steps. The first step, bypass of senescence, is achieved by inactivation of p53 and pRb by SV40 large T, human papillomavirus (HPV) E6 and E7, or by inhibition of p53 and pRb expression by the RNAi approach (or expression of dominant-negative mutants in the case of p53). The second step, immortalization, is achieved through the expression of hTERT. Alternatively, expression of HPV E6 or overexpression of Bmi-1, mutant p53, or ZNF217 can be used to induce immortalization of HMECs. The third step, anchorage-independent growth, can be achieved by SV40 small t mediated modulation of PI3K and/or other signaling pathways or by overexpression of activated Rac1 and AKT. The fourth step, full transformation, requires the introduction of activated H-ras, which can be substituted by Raf and Ral-GEFs. Although the current model systems have utilized the serial schemes depicted, other combinations and/or schemes of oncogene introduction are likely also to be effective. Adapted from Elenbaas [9], Zhao [10], and Rangarajan [11] and coworkers. HMEC, human mammary epithelial cell; HPV, human papillomavirus; hTERT, catalytic subunit of human telomerase; PI3K, phosphatidylinositol 3-kinase; Ral-GEF, Ral guanine nucleotide exchange factor; RNAi, RNA interference.

in a nude mouse model. Thus, future studies must focus on developing models that will lead to breast tumors that faithfully reproduce the pathological characteristics of human breast cancers.

Transgenic mouse models of breast cancers

Mouse models of breast cancers have provided a wealth of knowledge about the molecular pathways involved in breast cancers. Initial studies in these models used carcinogens to induce breast carcinomas [55]. Later studies targeted a wide variety of genes expressed under either the MMTV (mouse mammary tumor virus) or the WAP (whey acidic protein) promoter to target genes to the mammary gland. Importantly, such studies invariably produced breast adenocarcinomas in mice that resembled human breast cancers. These include viral proteins, such as SV40 large T, polyoma virus T antigen [56-58], or cellular proteins such as c-Myc, ErbB2/neu, cyclin D_1, cyclin E, ERs, mutant p53, c-Ha-ras, and Wnt-1 [59-63]. Recent studies have focused on mouse models with either a global or a mammary-specific knockout of specific genes to examine the function of obvious players, such as cell cycle related proteins and tumor suppressors, either by themselves or after these deficiencies were combined with transgenic neu or other oncogenes. For example, cyclin D_1-deficient mice are resistant to mammary carcinomas induced by c-neu/ErbB2 and Ha-ras but not to those induced by c-Myc or Wnt-1 [63]. These findings define a pivotal role for cyclin D_1 in selective mammary cancers in a mouse model and imply a functional role for cyclin D_1 overexpression in a subset of human breast cancers. In another study, Cre-mediated deletion of exons 3 and 4 of the mouse *Brca2* gene in mice with a loxP-modified and null *Brca2* allele resulted in high incidence of breast adenocarcinomas [64]. Similarly, the telomere attrition in aging telomerase-deficient and p53-mutant mice promoted the development of breast adenocarcinomas [65]. Another study showed that loss of Stat5a delays mammary cancer progression in a WAP-TAg transgenic mouse model [66].

Collectively, these models have defined a role for p53, pRb, BRCA1/2, cyclins, CDKs, ErbB2, c-Myc, Wnt-1, ER, and progesterone receptor in mammary cell growth and development of breast cancers. Finally, these different oncogenes and the pathways in which they work seem to target different progenitors or cell types in mammary gland to develop mammary tumors [67]. For example, the Wnt signaling pathway targets both luminal and myoepithelial cells, whereas Neu, H-Ras, and polyoma T antigen target only luminal epithelial cells [67]. The take-home lesson here is that the majority of these mouse models result in tumors that resemble human breast adenocarcinomas pathologically. The lack of development of adenocarcinomas from cells transformed in culture models may thus reflect the cell type that was used as the starting normal cell, rather than any peculiarity associated with the use of mouse as a host.

Molecular classification of breast cancers: cues from cell culture studies

A vast body of clinical literature indicates that breast tumors exhibit diverse phenotypes as judged by their distinct clinical course, pathological features, and responsiveness to various therapies. However, it has not been clear whether this diversity reflects cancers arising from distinct subtypes of HMECs. Consistent with such a possibility, several years ago we reported the presence of different subtypes of cells in reduction mammoplasty specimens and in milk that exhibited differential susceptibility to viral oncogenes [5,8]. Direct evidence for the conclusions derived from these cell culture studies was provided by recent work utilizing gene expression patterns in primary human breast cancers, using cDNA microarrays. These studies identified distinct gene expression profiles or molecular portraits based on which breast tumors could be subclassified into groups that appear to reflect the original cellular subtypes found in the mammary gland [12]. Five categories of breast cancers were described [12]: a basal epithelial-like group, an ErbB2-overexpressing group, a normal breast epithelial-like group, luminal epithelial cell type A, and luminal epithelial cell type B. A slightly different classification was proposed by Sotiriou and coworkers [68]. The breast tumors were first divided into ER-positive and ER-negative categories. The ER-negative tumors were further subgrouped into basal-like 1, basal-like 2, and ErbB2/neu tumors, whereas ER-positive tumors were subdivided into luminal-like 1, luminal-like 2, and luminal-like 3 subtypes. Sotiriou and coworkers also re-examined data from the study by Sorlie and coworkers [12] and suggested that luminal-like breast cancer could be classified as luminal A, B, and C subtypes corresponding to luminal-like 1, luminal-like 2, and luminal-like 3 subtypes.

Interestingly, survival analyses conducted in a subcohort of patients with locally advanced breast cancer uniformly treated in a prospective study showed significantly different outcomes for the patients belonging to the various groups, with the basal-like subtype correlating with worst outcome, followed by ErbB2 overexpressing, normal cell type and luminal cell type groups [12,68]. Interestingly, a significant difference in outcome for the two ER-positive groups was also noticed [68]. These studies strongly support the idea that many of the breast tumor subtypes may represent malignancies of biologically distinct cell types producing distinct disease entities that may require different treatment strategies. Importantly, these analyses provide a strong rationale for further definition of various mammary epithelial subtypes and expansion of immortalization and full transformation strategies to derive models that may faithfully reproduce the histological and molecular diversity encountered in human breast cancers.

Do breast cancers arise from stem cells?

Stem cells have enormous replicative potential and capacity for self-renewal, and give rise to different lineages of cells.

Although still a controversial notion, many cancers are thought to originate from cancer stem cells [69]. This idea has also attracted a great interest in the field of breast cancer research, and investigators have begun to examine whether there are mammary stem cells [13,17,27,70-73]. The cellular milieu of the mammary gland undergoes significant changes during pregnancy, lactation, and involution. These include bursts of proliferation of existing cells during pregnancy, continued differentiation during lactation, and apoptosis during involution at the end of the cycle. This cyclical behavior predicts the presence of a stem cell-like population in the mammary gland, which would meet the demand of a pregnancy cycle. The existence of adult mammary epithelial stem cells has therefore been proposed. Direct evidence for the existence of such cells has come from clear fat-pad transplantation, retroviral tagging, and X-chromosome inactivation studies in rodent model [13,16,17,70-73].

Recently, using various putative stem cell and cell surface markers, such as sialomucin (Muc), epithelial-specific antigen (ESA), various cytokeratins, ASMA, and CALLA or CD10, attempts have been made to identify the mouse and human mammary epithelial stem cells [13,27,70-73]. Using immunomagnetic cell sorting based on surface antigen markers (Muc and ESA) and subsequent immortalization with E6 and E7, Gudjonsson and coworkers [27] separated $Muc^-/ESA^+/K\text{-}19^+$ cells that were able both to self-renew and to give rise to Muc^-/ESA^+ epithelial cells and $ASMA^+$ myoepithelial cells, thus exhibiting characteristic of breast stem cells. Dontu and coworkers [13] isolated undifferentiated mammospheres from single cell suspensions of HMECs obtained by mechanical and enzymatic dissociations. Primary mammospheres can be further passaged to generate secondary mammospheres. Primary as well as secondary mammospheres were highly enriched in early progenitor or stem cells capable of differentiating along multiple lineages and of self-renewal. Immunostaining of these mammospheres showed the presence of CD10, α_6 integrin and K-5 on early progenitors, and ESA and K-14 on late progenitor cells [13]. However, MUC1, K-18, and ASMA were not expressed in cells present in mammospheres [13]. Detailed expression profiling of mammospheres suggests the presence of additional markers that are upregulated in mammospheres such as stem cell growth factor, hepatocyte growth factor antagonist, stem cell growth factor B and apolipoprotein E. Some markers are exclusively expressed in mammospheres such as FZD2 (frizzled homolog 2), glypican 4, interleukin-6, CXCR4 (CXC chemokine receptor), and FGFR1 (fibroblast growth factor receptor 1). Several genes that are expressed in mammospheres are also expressed in similar structures derived from other cell types (such as neurospheres formed by neural stem cells) [13].

Thus, culture of human HMECs in mammospheres may provide a tool with which to isolate and study mammary epithelial stem cells and their oncogenic susceptibilities. Based on the above and other related studies [13,17,27], the candidate mammary stem cells appear to be ESA^+, $MUC1^-$, α_6 $integrin^+$, and $CD10^+$, and the mammary stem cell niche appears to be at the suprabasal location within the luminal cell layer. Further work by other laboratories and adoption of the schemes employed by Gudjonsson [27] and Dontu [13] and their groups should help in determining the general feasibility of these novel approaches.

Apart from normal mammary stem cells, the possible existence of a breast cancer stem cell has been reported in the literature [74,75]. In a NOD/SCID xenotransplants model, Al-Hajj and coworkers [75] used four cell surface markers, CD44, CD24, ESA and B38.1 (a Breast/ovarian cancer specific marker), and lineage markers to sort different populations of breast cells from breast tumor tissues. All mice injected with $Lin^-/CD44^+/B38.1^+/CD24^{-/low}$ generated tumors, whereas none of the mice injected with $CD44^-/B38.1^-$ cells developed tumors. $Lin^-/CD44^+/B38.1^+$ fractions were further subdivided based on ESA expression. When used in numbers as low as 200, $Lin^-/ESA^+/CD44^+/CD24^{-/low}$ cells in xenotransplants generated tumors that were similar to initial tumors in term of phenotypic heterogeneity [75]. The presence of such a population in breast tumor tissue, which is able to self-renew and differentiate, supports the stem-cell model of breast tumorigenesis.

Conclusion

Our ability to culture and immortalize normal HMECs has provided a wealth of knowledge about the behavior of mammary cells and the genes involved in normal cell growth and oncogenesis. Characterization of these cells has provided novel markers that may permit early diagnosis and prognosis of breast cancers, and has yielded knowledge about potential precursor cells for breast cancers. Transformation analyses in cell culture models have also proven important to our understanding of the multistep nature of breast cancer. Transgenic mouse models have identified the roles played by various tumor suppressors, cell cycle proteins, and other proto-oncogenes in breast cancers. Recent studies using three-dimensional models have proven useful to our understanding of the normal and tumor mammary stem cells and the relationship of microenvironment to epithelial cell growth. Finally, using gene profiling, we have begun to appreciate that breast cancers do not originate only from luminal cells but also from basal and myoepithelial cells, and that there are subtypes of breast cancers that possibly originate from distinct normal precursors that have distinct clinical outcomes and may require different treatment strategies.

However, a number of critical questions remain. What are breast stem cells and what is their role in breast cancer? Are myoepithelial cells and basal cells similar or distinct? Why can we not culture most of the primary breast cancers? How can we develop transformed breast cells in culture that would give rise to breast tumors that resemble human breast cancer –

adenocarcinomas as opposed to poorly differentiated carcinomas? How do different subtypes of breast cancer originate?

In conclusion, experimental immortalization and transformation models have led to substantial progress in our understanding of the biology of breast cancer. Future studies in these model systems should go a long way toward elucidating the nature of breast cancer heterogeneity and thus facilitate the development of more individualized therapies for breast cancer patients.

Competing interests

The author(s) declare that they have no competing interests.

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What has senescence got to do with cancer?

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Cancer therapeutics are primarily thought to work by inducing apoptosis in tumor cells. However, various tumor suppressors and oncogenes have been shown to regulate senescence in normal cells, and senescence bypass appears to be an important step in the development of cancer. Cellular senescence limits the replicative capacity of cells, thus preventing the proliferation of cells that are at different stages of malignancy. A recent body of evidence suggests that induction of senescence can be exploited as a basis for cancer therapy.

Introduction

In simplistic terms, cancer is a disease of uncontrolled cell proliferation occurring at the wrong place at the wrong time, caused by oncogenic signals. To counter abnormal cell proliferation, a cell can either enter a quiescence-like growth arrest phase, undergo apoptosis, or senesce. These antiproliferative programs are induced by various tumor suppressors in response to potential oncogenic signals. In particular, p53 and pRB tumor suppressors are important mediators of quiescence, apoptosis, and senescence. The senescence phenomenon was first described by Hayflick and Moorhead in human fibroblasts (Hayflick and Moorhead, 1961). Senescent cells in culture are identified by large cell size, flat vacuolated morphology, inability to synthesize DNA, and the presence of the senescence-associated β-galactosidase (SA-β-gal) marker, which is detected by a colorimetric assay using X-gal as a substrate at pH 6.0 (Dimri et al., 1995). Using the SA-β-gal marker, and other senescence and aging biomarkers, several recent studies have demonstrated a role for cellular senescence in aging and cancer (reviewed in Itahana et al., 2004; Campisi, 2005; Lombard et al., 2005). While the exact role of senescence in aging is debatable, its role as a tumor suppressor mechanism is more widely accepted (reviewed in Smith and Pereira-Smith, 1996; Itahana et al., 2004).

The first indication of senescence being a tumor suppressor mechanism was obtained by somatic cell hybridization studies (Smith and Pereira-Smith, 1996). It was shown that a hybrid cell generated by fusion of a tumor (immortal) and a normal (mortal) cell always undergoes senescence in culture. Thus, the senescent phenotype is dominant over immortality, which is a recessive trait. Early studies also identified p53 and pRB as two principal regulators of senescence (Shay et al., 1991; reviewed in Itahana et al., 2004), further supporting the hypothesis of cellular senescence being a tumor suppressor mechanism (discussed below in detail). What are the senescence-initiating signals, and how are these signals transduced to induce a senescent phenotype? As described below, several studies implicate telomeric and nontelomeric signals in the induction of cellular senescence.

Senescence-initiating signals: Telomere is only the tip of the iceberg

The tips of a chromosome consist of telomere repeats, which are capped by telomere binding proteins (reviewed in Smogorzewska and de Lange, 2004). In human cells, progressive telomere shortening appears to be the primary cause of cellular senescence (Harley et al., 1990; reviewed in Kim et al., 2002). In most cases, the enzyme telomerase maintains telomere length. The catalytic subunit of telomerase (TERT), together with its RNA component (*TERC*), builds telomere repeats at the chromosome ends, which otherwise, owing to asymmetric DNA replication, are progressively lost (reviewed in Smogorzewska and de Lange, 2004; Kim et al., 2002). Most human somatic cells do not contain sufficient telomerase to maintain telomere length (Masutomi et al., 2003), resulting in telomere shortening after each round of cell division. Exogenous expression of telomerase can either increase or stabilize telomere length in normal human cells, and in some cases results in cell immortalization (Bodnar et al., 1998).

It is thought that telomere shortening beyond a certain limit or uncapping of telomere ends triggers a DNA damage response, thereby activating a checkpoint mediated by the p53 pathway, resulting in proliferation arrest. Signals emitted by the telomere dysfunction appear to be similar to double-strand DNA break (DSB)-induced signals (d'Adda di Fagagna et al., 2003; Takai et al., 2003; Gire et al., 2004). Senescent cells are enriched in the nuclear foci of phosphorylated histone H2AX (γ-H2AX), p53 binding protein 53BP1, NBS1, the phosphoS966 form of SMC1, and MDC1 (d'Adda di Fagagna et al., 2003). Moreover, inactivation of CHK1 and CHK2 checkpoint kinases can restore S phase progression in senescent cells (d'Adda di Fagagna et al., 2003; Gire et al., 2004). A more recent study using single cell parameters also suggested that telomere shortening-triggered senescence is a DNA damage response mediated by the ATM/ATR-p53-p21 pathway (Herbig et al., 2004). If cellular senescence induced by telomere dysfunction in human cells is a DNA damage response, then the DNA damaging agents and factors that mimic DNA damage should be able to induce cellular senescence. Indeed, DNA damaging agents and other cellular stresses can trigger a senescence-like phenotype (reviewed in Ben-Porath and Weinberg, 2004, Itahana et al., 2004), characterized by a large, flat morphology and the presence of the SA-β-gal marker (Figure 1).

Apart from DNA damage, certain undefined stress-causing signals also induce senescence. For example, senescence in cultured murine cells is thought to be due to stress induced by culture conditions (Sherr and De Pinho, 2000), which can be abrogated by decreasing oxygen concentration used in culturing these cells (Parrinello et al., 2003). In addition, oncogenic and mitogenic signals, such as activated H-RAS, can also induce senescence in primary cells (Figure 1). Thus, senes-

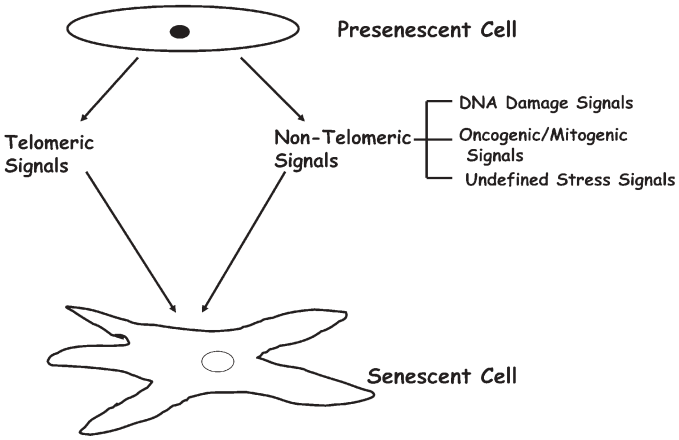

Figure 1. Presenescent cells undergo senescence in response to telomeric and nontelomeric signals

Telomeric signals such as telomere shortening and uncapping of telomere ends, as well as nontelomeric signals such as DNA damaging agents, oncogenic/mitogenic signaling, and undefined stress signals, induce senescence. Undefined stress signals are signals that come from a variety of sources, such as culture media. Senescent cells in culture are often identified by large, flat cell morphology, and stain positively for SA-β-gal marker.

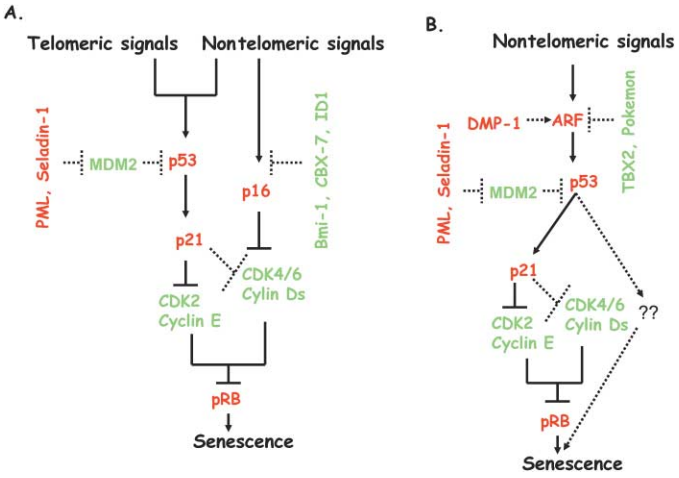

Figure 2. Telomeric and nontelomeric signals induce senescence via tumor suppressor pathways in human and mouse cells

Induction of p21 and p16 by senescence-inducing signals results in inhibition of activity of CDK2 and CDK4/6. Downregulation of activity of these pRB kinases leads to pRB hypophosphorylation, which results in cell cycle arrest during senescence. Regulators of senescence pathways in human and mouse cells include PML, MDM2, ID1, Bmi-1, CBX7, and Seladin-1. In mouse cells, tumor suppressor ARF is negatively regulated by two potential oncogenes, TBX2 and Pokemon, and positively regulated by DMP-1.

A: In human cells, telomeric and nontelomeric signals induce senescence primarily via the p53-p21-pRB pathway. Nontelomeric signals also induce the p16-pRB pathway of senescence in human cells.

B: Mouse cells undergo senescence via the ARF-p53-p21-pRB pathway in response to nontelomeric signals. Mouse cells do not senesce by the telomeric signal-induced pathway of senescence.

Solid lines indicate principal pathways of senescence, while dotted lines indicate auxiliary pathways that can modulate senescence. The red letters indicate tumor suppressors and growth inhibitors, while the green letters indicate oncogenes and growth promoters.

cence-inducing signals can be telomeric or nontelomeric (Figure 1). Senescence induced by nontelomeric signals is termed accelerated senescence, premature senescence, stress- or aberrant signaling-induced senescence (STASIS), or extrinsic senescence (Itahana et al., 2003, 2004). Senescence induced by nontelomeric signals may have evolved to protect organisms from acute signals that may cause cancer or other diseases resulting from faulty DNA replication.

Tumor suppressor pathways and senescence: Meant for each other

Regardless of the senescence-initiating signals (telomeric or nontelomeric), tumor suppressor pathways are critical for genesis and maintenance of the senescent phenotype in human and mouse cells. However, tumor suppressor pathways that pertain to senescence differ in human and mouse cells (Figure 2). In human cells, telomeric signals principally engage the p53-p21-pRB pathway (Figure 2A), while nontelomeric signals engage both the p53-p21-pRB and p16-pRB pathways (Figure 2A). In mouse cells, the ARF-p53-p21-pRB pathway is the dominant pathway of senescence (Figure 2B). However, pRB function in mouse cells can be substituted by pRB-related proteins p107 and p130 (reviewed in Itahana et al., 2004). It has also been suggested that p21 may not be the sole conduit of p53 during senescence induction in mouse cells (Pantoja and Serrano, 1999). Thus, other p53 targets may also participate in induction of senescence in mouse cells (Figure 2B). It is likely that these other p53 targets also contribute to senescence in human cells. Various other tumor suppressors and oncogenes also impinge upon these pathways of senescence and modulate them accordingly (Figure 2) (described below). Nonetheless, p53 and pRB remain the two main regulators of cellular senescence in human and mouse cells.

p53, the master regulator of senescence

Depending on the severity of damage to the genome, p53 can activate genetic programs that halt cell proliferation transiently (G1 and G2 cell cycle arrest) or permanently (senescence), or eliminate the cell altogether (apoptosis). Evidence for its role in senescence comes from several studies. First, as described earlier, it has been clearly shown that telomere shortening or dysfunction induces a DNA damage response mediated by p53. Second, abrogation of the p53-p21 pathway by various strategies can bypass senescence in human and mouse cells (Shay et al., 1991; Brown et al., 1997; Dirac and Bernards, 2003; Beausejour et al. 2003). Third, enforced expression of p53 or p21 in certain cell types can induce a senescence-like phenotype (Itahana et al., 2001). Finally, a variety of stimuli induce senescence in a p53/p21-dependent manner (Itahana et al., 2001, 2004).

It is interesting to note that studies using DNA tumor viruses to inactivate p53 suggested that p53 inactivation only extends the replicative life span, and complete abrogation of senescence leading to crisis requires pRB inactivation as well (Shay et al., 1991). However, more recent studies using either somatic cell knockout or an RNAi (RNA interference) approach suggest that p53 and p21 inactivation can lead to complete abrogation of senescence and induction of a crisis-like phenotype (Brown et al., 1997, Dirac and Bernards, 2003, and our unpublished data). How does p53 induction during senescence cause cell cycle arrest? Most studies suggest that p21 induction by p53 inhibits CDK2/Cyclin E activity. Activity of CDK4/Cyclin Ds can also be inhibited by p21. Inhibition of activity of CDKs by p21 results in hypophosphorylation of pRB, which very likely medi-

ates cell cycle arrest during senescence (Figure 2) (Itahana et al., 2004).

The role of p53 regulators in senescence

Tumor suppressor p53 is positively or negatively regulated by a plethora of factors (Figure 2) (reviewed in Vousden, 2002). The E3 ubiquitin ligases MDM2, PIRH2, and COP1 negatively regulate p53 by targeting it for proteosome-mediated degradation (reviewed in Lu, 2005). On the other hand, p53 is positively regulated by ARF ($p14^{ARF}$ in human or $p19^{ARF}$ in mouse), PML, PTEN, NPM, p33ING1, and other potential tumor suppressors, which posttranslationally stabilize p53 (Weber et al., 1999, Leung et al., 2002, Freeman et al., 2003, Bernardi et al., 2004, Kurki et al., 2004). In principle, these positive and negative regulators of p53 can also impact cellular senescence (Figure 2).

Indeed, p33ING1 is known to be overexpressed in senescent cells (Garkavtsev and Riabowol, 1997), and its overexpression can induce senescence in proliferating cells (Goeman et al., 2005). Similarly, nucleophosmin (NPM) overexpression was shown to induce senescence in a p53-dependent manner (Colombo et al., 2002). On the other hand, NPM also interacts with ARF tumor suppressor and inhibits its function (reviewed in Zhang, 2004). Interestingly, NPM is mutated in a large number of cases of acute myelogenous leukemia (AML) in humans (Falini et al., 2005). It is tempting to speculate that NPM mutations in these cases may be related to its possible role in senescence.

PML and p53 pathway of senescence

PML binds MDM2 and sequesters it into the nucleolus (Bernardi et al., 2004), thus protecting p53 from proteosome-mediated degradation. As a result, overexpression of PML, and accumulation of PML and MDM2 in the nucleolus after DNA damage, results in p53 stabilization (Bernardi et al., 2004). PML is upregulated during cellular senescence (Ferbeyre et al., 2000), and induces premature senescence in response to oncogenic H-RAS by promoting p53 acetylation (Pearson et al., 2000). Moreover, human SIR2, which deacetylates p53, inhibits PML- and p53-induced premature senescence, further confirming the role of PML as a senescence-regulatory protein (Langley et al., 2002).

More detailed studies have recently suggested that PML isoform IV, when overexpressed, induces senescence in human fibroblasts in a pRB-dependent manner (Mallette et al., 2004; Bischof et al., 2005), and that the cytoplasmic isoform of PML induces cellular senescence in response to TGF-β (Lin et al., 2004). Collectively, these studies suggest that the PML tumor suppressor contributes to cellular senescence in human and mouse cells.

ARF, an upstream regulator of p53 pathway and senescence

The INK4a/ARF locus encodes $p16^{INK4a}$ and ARF, which regulate pRB and p53 pathways of senescence and tumor suppression, respectively (reviewed in Lowe and Sherr, 2003; Sharpless, 2005). As indicated before, the ARF-p53 pathway is the major pathway of senescence in mouse cells. ARF is overexpressed in cultured senescent mouse embryo fibroblasts (MEFs) and upregulated during premature senescence induced by onocogenic signals such as activated H-RAS (Kamijo et al., 1999; reviewed in Lowe and Sherr, 2003; Sharpless, 2005) (described below in detail). Although ARF overexpression can also promote senescence in human cells (Dimri et al., 2000; Wei et al., 2001), it may not be a major regulator of senescence in human cells (Wei et al., 2001; reviewed in Sharpless, 2005).

ARF is thought to antagonize MDM2-mediated ubiquitination of p53 through translocation of MDM2 to the nucleolus, thereby stabilizing p53 (reviewed in Sherr and Weber, 2000). Although it can also inhibit cell proliferation by p53-independent pathways (reviewed in Cleveland and Sherr, 2004), ARF probably promotes senescence by regulating the p53 pathway (Dimri et al., 2000; Wei et al., 2001). The specific transcriptional repressors of ARF include TBX2, which was identified in a senescence bypass screen (Jacobs et al., 2000), and the protooncogene Pokemon, which can bind to the ARF promoter and repress its transcription (Maeda et al., 2005). MEFs lacking *Pokemon (Zbtb7)* exhibit constitutive upregulation of $p19^{ARF}$ and undergo premature senescence (Maeda et al., 2005). Importantly, TBX2 and Pokemon are aberrantly overexpressed in a subset of breast cancers and lymphomas, respectively (Jacobs et al., 2000; Maeda et al., 2005), suggesting a possible role for these ARF regulators in senescence in human cells as well.

pRB, the second regulator of cellular senescence

In contrast to p53, the role of pRB in cellular senescence is less clear (Itahana et al., 2004). Earlier studies using DNA tumor viruses that bind and inactivate pRB clearly indicate that pRB cooperates with p53 during cellular senescence (Shay et al., 1991). More recent studies suggest that perhaps pRB is, in some instances, as important as p53 in inducing cellular senescence. Using the Cre-Lox system to delete *pRB* in senescent MEFs, Sage et al. showed that the loss of pRB is sufficient for cell cycle entry and the reversal of cellular senescence (Sage et al., 2003). In human fibroblasts, loss of pRB by targeted disruption of one copy followed by the spontaneous loss of its other allele results in bypass of replicative senescence and a crisis-like phenotype similar to that induced by the abrogation of the p53-p21 pathway (Wei et al., 2003). Inactivation of an exogenously introduced temperature-sensitive pRB in senescent SAOS-2 cells also results in S phase reentry (Alexander et al., 2003). Collectively, these data suggest that pRB maintains cell cycle arrest during senescence in human and mouse cells.

It is very well established that pRB remains constitutively hypophosphorylated in senescent cells (Stein et al., 1999), suggesting downregulation of the activity of pRB kinases during senescence. The $p16^{INK4a}$, an inhibitor of CDK4 and CDK6 activity, is upregulated during senescence in human fibroblasts (Alcorta et al., 1996; Stein et al., 1999) and M0 senescence in human mammary epithelial cells (Wong et al., 1999). Thus, high p16 very likely accounts for the hypophosphorylation of pRB in senescent cells. Consistent with this hypothesis, $p16^{INK4a}$ and its upstream regulators such as Bmi-1, CBX7, ID1, and Ets-1 regulate senescence (Jacobs et al., 1999; Ohtani et al., 2001; Itahana et al., 2003; Gil et al., 2004) in a pRB-dependent manner (Figure 2).

Our recent data suggested that p16 upregulation during senescence is not nearly as universal as previously thought (Itahana et al., 2003). In certain fibroblast strains such as the commonly used WI-38 strain, p16 is clearly upregulated, while in other strains such as BJ fibroblasts, it is only minimally expressed under normal culture conditions (Itahana et al., 2003). The fibroblasts that contain high or low p16 differ in their propensity to reverse senescence by the inactivation of p53 pathway (Beausejour et al., 2003). Senescent WI-38 fibroblasts, which contain high p16, are essentially resistant to inactivation of p53 pathway, and cannot be induced to reenter cell cycle by

inactivation of p53. In other fibroblasts, which contain undetectable p16, senescence can by reversed by p53 inactivation (Beausejour et al., 2003). Fibroblast strains that accumulate p16 differentially during senescence also differ in the presence or absence of senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003). SAHF are heterochromatic DNA regions, where pRB was found to be colocalized (Narita et al., 2003). SAHF are present only in human fibroblasts that contain high p16 during senescence, suggesting that p16 plays a role in generating SAHF. Interestingly, senescence in SAHF-containing fibroblasts is irreversible (Beausejour et al., 2003). Thus, SAHF possibly contribute to the stable repression of proliferation-associated genes mediated by E2F-pRB complexes to enforce the senescent phenotype.

Recently, using a p16 knockdown strategy, it was shown that p16 downregulation may not be functionally equivalent to pRB inactivation (Wei et al., 2003), leaving the possibility that other CDK inhibitors might play a surrogate role in cellular senescence. In support of this hypothesis, overexpression of various CDK inhibitors is known to induce a senescent phenotype (McConnell et al., 1998). Since p21 induction can also lead to the inhibition of pRB phosphorylation by inhibiting CDK2/Cyclin E activity (Figure 2), p16 and p21 are likely to cooperate to keep pRB in a hypophosphorylated form during senescence. Alternatively, it is possible that p21 initially and temporarily keeps pRB in its inhibitory form, while p16 ensures permanent hypophosphorylation of pRB, which practically makes senescence irreversible (Beausejour et al., 2003).

Oncogenic and mitogenic signals induce senescence: A failsafe mechanism

As senescence is regulated by various tumor suppressors, it could function as a natural barrier to tumorigenesis. This hypothesis could be directly tested by subjecting a normal cell to potential oncogenic or mitogenic stimuli. Indeed, activated H-RAS (V12) was found to induce premature senescence in primary rodent and human cells (Serrano et al., 1997). Depending on the cellular context, induction of senescence by oncogenic signals such as activated H-RAS depends on either or both p53 and $p16^{INK4a}$ tumor suppressor proteins (Palmero et al., 1998; Lin and Lowe, 2001).

Since RAS signaling involves the RAF-MEK-ERK pathway, it is conceivable that other components of this pathway can also induce premature senescence. Indeed, oncogenic RAF and constitutive expression of mitogen-activated protein (MAP) kinase mimic RAS-induced premature senescence in IMR90 fibroblasts (Zhu et al., 1998; Lin et al., 1998). Furthermore, during oncogenic RAS-induced premature senescence, the RAF-MEK-ERK pathway activates p38 MAPK, and inhibition of p38 activity results in a failure to induce premature senescence by activated RAS (Wang et al., 2002). Constitutively active MKK3 and MKK6, which activate p38 MAPK by phosphorylation, can also induce premature senescence by upregulating p53 and $p16^{INK4a}$ in human fibroblasts (Wang et al., 2002).

Apart from p53, PML, ARF, and $p16^{INK4a}$, other proteins are also likely to mediate the H-RAS response. For example, recently, a genetic suppressor element (GSE) screen identified *Seladin-1* as a target gene that is involved in H-RAS-induced premature senescence (Wu et al., 2004). Interestingly, *Seladin-1* encodes an oxidoreductase enzyme involved in cholesterol metabolism (Wu et al., 2004). Further studies on Seladin-1 demonstrated that it is an effector of RAS-induced reactive oxygen species (ROS) signaling (Wu et al., 2004).

A careful analysis of oncogenic, mitogenic, and other hyperproliferative signals is likely to reveal more cases of premature senescence induction by such signals in primary cells, which in all likelihood represents a failsafe mechanism. For example, similar to activated H-RAS expression, E2F1 overexpression, a potent mitogenic signal, leads to premature senescence in normal human fibroblasts (Dimri et al., 2000). Premature senescence induction by E2F1 depends on the p53 status of the cells and is mediated by transcriptional induction of $p14^{ARF}$ by E2F1 (Dimri et al., 2000). Constitutive overexpression of E2F3 also results in induction of senescence in a transgenic mouse model and cultured MEFs (Denchi et al., 2005). In this study, it was found that a sustained E2F activity, which provides a hyperproliferative signal, induced senescence-like features in mouse pituitary gland (Denchi et al., 2005). This report is significant because it indicates that senescence induction by hyperproliferative signals is not merely an in vitro phenomenon.

Recently, it was shown that the overexpression of oncogenic ERBB2 also upregulates p21 and induces premature senescence in MCF-7 cells (Trost et al., 2005). The induction of ERBB2 in this setting causes p53-independent p21 upregulation and premature senescence, which can be reversed by the inhibition of p38 MAPK or functional inactivation of p21 by antisense oligonucleotides (Trost et al., 2005).

Beyond senescence: The road to the cancer highway

Primary cells induce senescence in response to potential oncogenic signals. What happens when the induction of senescence fails to occur due to malfunction of tumor suppressors? Cancer is a multistep process; hence, abrogation of senescence alone does not lead to tumor formation. Nevertheless, the road starts from here. Recent elegant studies from the laboratories of Weinberg and Hahn clearly demonstrate that the first step in creating an in vitro model of human cancer involves the abrogation of cellular senescence (Hahn et al., 1999; reviewed in Boehm and Hahn, 2005). These studies show that a combination of SV40 large T, small t, hTERT, and H-RAS is able to transform a variety of normal human cell types such as fibroblasts, embryonic kidney cells, mammary epithelial cells, ovarian epithelial cells, and endothelial cells (Boehm and Hahn, 2005).

Abrogation of senescence can be achieved by SV40 large T, a combination of HPV oncoproteins E6 and E7, E1A and MDM2 coexpression, or the use of short inhibitory (sh) RNA against pRB and p53 ((Hahn et al., 1999; Seger et al., 2002; Voorhoeve and Agami, 2003; Boehm and Hahn, 2005). Since the INK4a/ARF locus is an upstream regulator of both pRB and p53 (reviewed in Lowe and Sherr, 2003; Sharpless, 2005), various mutations in this locus presumably can also substitute SV40 large T function in transformation assays, albeit at a lower efficiency depending on the nature of the mutation. For example, a combination of hTERT and H-RAS or c-MYC is sufficient to transform Leiden HDFs (human diploid fibroblasts), which bear an INK4a/ARF mutation resulting in $p16^{INK4a}$ deficiency (Drayton et al., 2003). Similarly, a combined knockdown of $p16^{INK4a}$ and p53 by the RNAi approach, together with SV40 small t, hTERT, and H-RAS, causes transformation of normal human fibroblasts (Voorhoeve and Agami, 2003).

Compared to human cells, murine cells are clearly less rigid in terms of requirements for different genetic mutations for transformation (reviewed in Rangarajan and Weinberg, 2003).

Nevertheless, bypass of senescence is also essential for murine cell transformation. Established immortal MEF cell lines have often lost p53 or $p19^{ARF}$. MEFs deficient in $p19^{ARF}$ are highly susceptible to oncogenic transformation and resistant to H-RAS-induced senescence (Sharpless et al., 2004). The H-RAS induced senescence can also be abrogated by other potential oncogenes, such as hDRIL and BCL6 (Peeper et al., 2002; Shvarts et al., 2002), which indirectly affects pRB and p53 pathways of senescence. For example, BCL6 overrides p53 pathway by inducing cyclin D1 expression (Shvarts et al., 2002), while hDRIL targets pRB pathway by binding to E2F1 (Peeper et al., 2002). These potential oncogenes strongly cooperate with H-RAS to transform mouse and human cells. Similarly, downregulation of Seladin-1 expression by sh RNA, together with hTERT and H-RAS, not only overcomes RAS-induced senescence, but also transforms human fibroblasts (Wu et al., 2004). Deficiency of transcription factor DMP-1, which works upstream of ARF, also promotes H-RAS-induced transformation in MEFs (Inoue et al., 2000; Sreeramaneni et al., 2005). Thus, malfunction of senescence and tumor suppressor pathways facilitate transformation by oncogenes in human and mouse cells.

Senescence in cancer treatment: Putting a roadblock in the cancer highway

So far, most studies have suggested that the failure of senescence-induction pathways in conjunction with activated oncogenes possibly leads to tumor formation in vivo. However, an important question is, can senescence still be induced in tumors to stop further growth? Indeed, several recent studies have shown that chemotherapeutic drugs and radiation can induce senescence in tumor cells (reviewed in Roninson, 2003). Quite often, tumors develop resistance to chemotherapeutic drug-induced apoptosis. Senescence induction in such cases could serve as a backup plan to inhibit the growth of tumor cells. Indeed, recently, it was reported that a combined treatment of cells with pan caspase inhibitor (Q-VD-OPH) and doxorubicin greatly accelerates senescence and leads to the reversal of drug resistance in several tumor cell lines (Zheng et al., 2004).

The induction of senescence in cultured tumor cells by DNA-damaging agents is encouraging, but the most important question is, does senescence response to chemotherapeutic drugs occur in vivo, and if so, does the therapy-induced senescence contribute sufficiently to the therapeutic efficiency? Recent studies provide compelling evidence that cellular senescence can indeed be induced in vivo by chemotherapeutic drugs. In the first study, te Poele et al. stained newly sectioned archival breast tumors from patients who had undergone a chemotherapy regimen for the SA-β-gal marker and p53 and $p16^{INK4a}$ proteins (te Poele et al., 2002). While normal tissues adjacent to the tumors were devoid of SA-β-gal, importantly, 15 out of 36 (41%) tumors stained positive for SA-β-gal marker (te Poele et al., 2002). Authors also showed that the tumor sections from the patients that did not receive chemotherapy stained positively for SA-β-gal only in 10% of the cases, and this staining was in few isolated cells compared to many intense patches of staining present in treated tumor sections. Furthermore, intense SA-β-gal staining was correlated with high $p16^{INK4a}$, a protein known to be upregulated during senescence. Although authors did not attempt to correlate SA-β-gal staining to survival, it was speculated that senescence induction by chemotherapy results in a stable disease rather than the regression of tumor, a situation often noticed during treatment by cytotoxic drugs in patients (te Poele et al., 2002). The induction of accelerated or premature senescence during chemotherapy treatment of human lung cancer in vivo was also recently demonstrated (Roberson et al., 2005). Although the sample size in this study was very small, it was found that two of the three patients treated with carboplatin and taxol intensely expressed SA-β-gal, while another three samples from untreated patients showed no significant SA-β-gal staining (Roberson et al., 2005).

A study by Schmitt et al. also provides a clear evidence for the role of senescence in cancer chemotherapy in a transgenic mouse model (Schmitt et al., 2002). The authors showed that CTX (cyclophosphamide), a chemotherapy drug, is able to engage a senescence program when apoptosis is inhibited by Bcl2 overexpression during Eμ-Myc-induced lymphoma in wild-type p53-containing transgenic mice. As a result, Bcl2 and wild-type p53-expressing lymphoma did not progress, and the mice had a better prognosis after CTX treatment (Schmitt et al., 2002). Induction of the senescence-like phenotype was also observed in rat mammary tumors undergoing treatment with chemopreventive agents (Christov et al., 2003). Thus, chemotherapeutic drugs can induce a senescence-like stage in vivo and in vitro by upregulating p53 and/or $p16^{INK4a}$.

More than 90% of tumors contain readily detectable telomerase activity (Kim et al., 1994). Telomerase, and various telomerase-regulatory and telomere binding proteins, are thought to regulate telomere length in a cell (Smogorzewska and de Lange, 2004). In principle, telomerase and telomerase-regulatory proteins can be targeted to cause telomere dysfunction and induce apoptosis or senescence in precancerous and tumor cells (Shay and Wright, 2002). Indeed, several telomerase inhibitors are known to induce senescence or apoptosis in tumor cells (Damm et al., 2001, Seimiya et al., 2002, Riou et al., 2002, Kim et al., 2003; Preto et al., 2004). Recently, inhibition of tankyrase 1, which poly (ADP-ribosyl)ates TRF1, was shown to cooperate with a telomerase inhibitor MST-312 to cause telomere shortening and rapid cell death (Seimiya et al., 2005). Combined treatment of cells with telomerase inhibitors and other regulators of telomerase may be helpful when tumors develop resistance to telomerase inhibitors alone (Seimiya et al., 2005).

Senescence may promote tumorigenesis: An unwanted side effect

An interesting question is what could be the side effect of the induction of senescence by chemotherapeutic drugs, and what could compromise the efficacy of treatment by these drugs? It has been proposed that senescence in some settings may actually promote tumor progression; possibly by secreting certain matrix metalloproteases, growth factors, and cytokines (Krtolica et al., 2001; reviewed in Campisi, 2005). In particular, senescent fibroblasts were shown to facilitate tumorigenesis by immortal premalignant epithelial cells (Krtolica et al., 2001).

It is important to note that in a therapeutic setting, both normal host and tumors cells are being treated with senescence-inducing agents. Although induction of senescence in the tumor cell itself is unlikely to promote any more tumorigenicity, its induction in normal cells by chemotherapeutic drugs may facilitate tumorigenesis by cells that are not yet fully tumorigenic. Based on a recent study by te Poele et al., normal cells adjacent to tumors appear to be less receptive to senescence induction by chemotherapeutic drugs (te Poele et al., 2002). Clearly, more

studies are needed to address this question of facilitation of tumorigenesis by senescent cells during chemotherapy of tumors in patients.

Concluding remarks

In this review, I have highlighted the role of senescence in cancer. Senescence is not only a normal physiological response to accrued cell divisions in culture, but is also very likely a response to potential oncogenic events that a cell might encounter. Thus, a senescence response is elicited by DNA damage and oncogenic and mitogenic signals. In this scenario, senescence acts as a failsafe mechanism. Mutations in p53 and/or pRB are a common occurrence in various cancers. INK4a/ARF locus, which is an upstream regulator of p53 and pRB, is also a target of many somatic and genetic mutations. These mutations often cause a bypass of cellular senescence and cooperate with other oncogenes in transformation assays, thus attesting to the importance of senescence in cancer. The take-home message is that a tumor will not come into existence unless it has bypassed senescence. The fascinating part here is that despite the fact that tumors have bypassed the common pathway(s) of senescence, they retain the ability to undergo senescence in response to treatment with a variety of therapeutic agents.

Several in vitro studies suggest that chemotherapeutic drugs can induce a senescence-like phenotype. A limited number of in vivo studies in the human and mouse models provide proof of principle for the concept of induction of accelerated senescence by chemotherapeutic drugs. Clearly, more studies are needed to substantiate these findings, particularly in human cancers. Further caution is also warranted, because senescence induction in normal cells can facilitate tumor progression under certain circumstances. Thus, a critical degree of senescence may be the prerequisite for successful treatment using chemotherapeutic drugs. Although it is still unclear how much therapy-induced senescence can contribute to the therapeutic efficiency, it is likely to result in a stable disease rather than the regression of tumors. Apart from chemotherapeutic drugs, senescence induction can also be achieved by small molecules that directly target senescence-regulatory genes and under- or overexpression of various genes involved in senescence. In summary, senescence has a lot to do with cancer development, and a better understanding of the senescence induction pathways will greatly contribute to the development of effective cancer treatment strategies.

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Seimiya, H., Oh-hara, T., Suzuki, T., Naasani, I., Shimazaki, T., Tsuchiya, K., and Tsuruo, T. (2002). Telomere shortening and growth inhibition of human cancer cells by novel synthetic telomerase inhibitors, MST-312, MST-295, and MST-199. Mol. Cancer Ther. *1*, 657–665.

Seimiya, H., Muramatsu, Y., Ohishi, T., and Tsuruo, T. (2005). Tankyrase 1 as a target for telomere-directed molecular cancer therapeutics. Cancer Cell *7*, 25–37.

Seger, Y.R., Garcia-Cao, M., Piccinin, S., Cunsolo, C.L., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. (2002). Transformation of normal human cells in the absence of telomerase activation. Cancer Cell *2*, 401–413.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593–602.

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Stein, G.H., Drullinger, L.F., Soulard, A., and Dulic, V. (1999). Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. Mol. Cell. Biol. *19*, 2109–2117.

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Takai, H., Smogorzewska, A., and And de Lange, T. (2003). DNA damage foci at dysfunctional telomeres. Curr. Biol. *13*, 1549–1556.

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Wang, W., Chen, J.X., Liao, R., Deng, Q., Zhou, J.J., Huang, S., and Sun, P. (2002). Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. Mol. Cell. Biol. *22*, 3389–3403.

Weber, J.D., Taylor, L.J., Roussel, M.F., Sherr, C.J., and Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. Nat. Cell Biol. *1*, 20–26.

Wei, W., Hemmer, R.M., and Sedivy, J.M. (2001). Role of p14(ARF) in replicative and induced senescence of human fibroblasts. Mol. Cell. Biol. *21*, 6748–6757.

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Wong, D.J., Foster, S.A., Galloway, D.A., and Reid, B.J. (1999). Progressive region-specific de novo methylation of the p16 CpG island in primary human mammary epithelial cell strains during escape from M(0) growth arrest. Mol. Cell. Biol. *19*, 5642–5651.

Wu, C., Miloslavskaya, I., Demontis, S., Maestro, R., and Galaktionov, K. (2004). Regulation of cellular response to oncogenic and oxidative stress by Seladin-1. Nature *432*, 640–645.

Zhang, Y. (2004). The ARF-B23 connection: Implications for growth control and cancer treatment. Cell Cycle *3*, 259–262.

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Zhu, J., Woods, D., McMahon, M., and Bishop, J.M. (1998). Senescence of human fibroblasts induced by oncogenic *Raf*. Genes Dev. *12*, 2997–3007.

CURRICULUM VITAE

PERSONAL DATA

Name: Goberdhan P. Dimri, Ph.D.

Professional Title: Assistant Professor of Medicine

Work Address: Department of Medicine,
ENH Research Institute, 1001 University Place, Evanston, IL 60201.
Phone: Office (224) 364-7521; Cell (224) 392-1770
Fax: (224) 364-7402
Email: gdimri@enh.org; g-dimri@northwestern.edu

Home Address: 240 Valley View Drive, Wilmette, IL 60091
Phone: (847) 920-1024; Email: gdimri@aol.com

Citizenship Status: Naturalized Citizen

EDUCATION:

Year	Degree	Discipline	University
1984	M.Sc.	Life Sciences	J. N. University, New Delhi, India
1985	M.Phil.	Environmental Sciences	J. N. University, New Delhi, India
1990	Ph.D.	Environmental Sciences	J. N. University, New Delhi, India

POSTDOCTORAL RESEARCH TRAINING:

1989-1991: Postdoctoral Fellow, Department of Molecular and Cell Biology, University of California, Berkeley, CA

1991-1994: Postdoctoral Fellow, Department of Cell and Molecular Biology, Lawrence Berkeley National Laboratory, University of California, Berkeley, CA

HONORS AND AWARDS:

1982-1984:	Merit scholarship, J. N. University, New Delhi, India
1984-1986:	Junior Research Fellowship, University Grants Commission, India
1986-1989:	Senior Research Fellowship, University Grants Commission, India
1994:	Travel Award: from National Institute on Aging and Gordon Conferences to attend Gordon Conference on Biology of Aging

FACULTY APPOINTMENTS:

1999-2003:	Assistant Professor, Department of Radiation Oncology, New England Medical Center, Tufts University, Boston, MA
2003-Present:	Assistant Professor (Tenure-Track) of Medicine, Feinberg School of Medicine, Northwestern University, Evanston, IL

OTHER PROFESSIONAL APPOINTMENTS:

1989:	Scientist B, Genetic Engineering Unit, J. N. University, New Delhi, India
1995-1999:	Scientist, Department of Cell and Molecular Biology, Lawrence Berkeley National Laboratory, University of California, Berkeley, CA
1999-2003:	Special Scientific Staff, Department of Radiation Oncology, Division of Radiation and Cancer Biology, New England Medical Center, Boston, MA
2003-Present:	Senior Scientist, Division of Cancer Biology, Department of Medicine, ENH Research Institute, Evanston, IL
2003- Present:	Professional Staff, Evanston Northwestern Healthcare, Evanston, IL
2003-Present:	Member, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL

SUMMARY OF TEACHING EXPERIENCE:

Following trainees were trained or being trained under my supervision in various aspects of Cell and Molecular Biology, and research related to Cancer and Aging:

Trainees during Postdoctoral Fellow position:

1991-1994:	Trishia Chandra, Research associate, Lawrence Berkeley National Laboratory, Berkeley, CA
1991-1993:	Ying Lee, Research associate, Lawrence Berkeley National Laboratory, Berkeley, CA
1994-1999:	Meilleen Acosta, Research associate, Lawrence Berkeley National Laboratory, Berkeley, CA

1998-1999: Ying Zou, Research associate, Lawrence Berkeley National Laboratory, Berkeley, CA

Trainees during Independent Investigator position:

2000-2002: Dr. Jose-Luis Martinez, Postdoctoral Fellow, New England Medical Center, Boston, MA

2002-2003: Dr. Suresh Kumar, Postdoctoral Fellow, New England Medical Center, Boston, MA

2002-2004: Dr. Libing Song, Postdoctoral Fellow, New England Medical Center, Boston, MA; Postdoctoral Follow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2003-2004: Dr. Yvonne Fondufe, Research Associate, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2003-2004: Dr. Natesan Sankar, Research Associate, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2003-Present: Dr.Sonal Datta, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2004-Present: Dr. Ajay Kumar, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2005-Present: Dr. Mukesh Gandhari, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2005-Present: Dr. Wei Jian Guo, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2005- Present: Prashant Boomi Reddi, Research Assistant, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2006: Hema, RamKumar, PreMed. Underegraduate Student, Northwestern University

PROFESSIONAL AND SCIENTIFIC ACTIVITY:

Patents:

1998: U.S. Patent 5,491,069 and 5,795,728; "Biomarkers of Cell Senescence"

Review and Editorial Services:

1994- Present:	Peer reviewed papers for Exp. Cell Res. & J. Geron.
2000- Present:	Peer reviewed papers for Cancer Res. & Biogerontology
2003- Present:	Peer reviewed papers for Cancer Letters & J. Biol. Chem.
2004- Present:	Peer reviewed papers for J. Mol. Cell Life Sciences, Mech. Aging and Development, J. Clin. Investigation, J. Lab Investigation, Mol. Cell Biol., Life Sci., Analytical Biochem., Biotech., Apoptosis.
2001- Present:	Biogerontology, Editorial Board Member

Grant Review Panels:

2005:-Present	NIH Study Section member "ZRG1 F05, Cell Biology Fellowships"
2005:	Peer Review Panel Member, Cell Biology #3, USAMRMC, 2005 Breast Cancer Research Program

International Peer Review Services:

2005:	Peer reviewed grant proposals for "Austrian Science Fund (FWF)", Vienna, Austria
2005:	Reviewer, Doctoral Scholarship Program of the Austrian Academy of Sciences, Vienna, Austria
2006:	Peer reviewed grant proposals for "The Italian Association for Cancer Research" (AIRC), Milan, Italy
2006:	Peer reviewed grant proposals for the "Netherlands Organization for Scientific Research (NOW, the Dutch Research Council)"

Other Review Services:

2005:	Reviewed Pilot Research Projects for "Center for Genetics and Molecular Medicine', Univ. of Louisville, KY
2006:	External Evaluator for Faculty Appointments (Tenure- Track Assistant and Associate Professor), UCLA School of Dentistry, Los Angeles, CA

Memberships:

1995- Present:	American Society for Cell Biology
1994- Present:	American Association for the Advancement of Science
1996- Present:	American Society for Microbiology
2005-Present:	American Association for Cancer Research

OTHER ASSIGNMENTS/DUTIES:

2005- Present:	"Invention Disclosure and Patent Application Review Committee", ENH Research Institute, Evanston, IL

RESEARCH GRANTS/CONTRACTS:

Active:

1.

Title: Role of p53 in Mammary Epithelial Cell Senescence
Role: PI
Funding Agency: US ARMY Medical Research and Material Command
Direct cost: $221,549
Duration: 05/01/02-04/30/07

2.

Title: Role of Bmi-1 in Telomerase Regulation and Breast Cancer

Role: PI
Funding Agency and Grant Number: NIH, 1RO1 CA094150
Total Direct Cost: $1,000,000
Duration: 09/17/03-08/30/08

Pending:

1. Title: Secretome of breast cancer invasion
Role: PI
Funding Agency and Grant Number: US ARMY Medical Research and Material Command
Total Direct Cost: $75,000

2. Title: Bmi-1 Oncoprotein and Chemoresistance in Breast Cancer Cells
Role: PI
Funding Agency and Grant Number: US ARMY Medical Research and Material Command
Total Direct Cost: $300,000

3. Title: A genetic screen for candidate tumor suppressors in human mammary epithelial cells
Role: PI
Funding Agency and Grant Number: US ARMY Medical Research and Material Command
Total Direct Cost: $300,000

Past:

1. Title: Senescence-specific Promoter Vectors
Role: PI
Funding Agency and Grant Number: NIH, AG165851-01
Total Direct Cost: $50,000
Duration: 03/01/99-02/28/01

2. Title: Polycomb Proteins and Breast Epithelial Cell Transformation
Role: PI
Funding Agency and Grant Number: USAMRMC- BC032256
Direct Cost- $75,000/year
Duration: 07/01/04-06/30/05

Others

Role- Consultant
Title: Stress and Aging among Parental Caregivers of Childhood Brain Tumor Survivors (PI- Witt, Whitney P., M.PH., Ph.D., Buehler Center on Aging, Northwestern University)
Funding Agency- NIH
Direct Cost/Duration- Pending

PUBLICATIONS:
Refereed Journal Articles:

1. Phadnis, S. H., **Dimri, G. P.** and H. K.Das (1988) Segregation characteristics of multiple chromosomes of *Azotobacter vinelandii*. **J. Genet.** 67: 37-42.

2. **Dimri, G. P.,** Roy, K. B. and H. K.Das (1988) Cloning of ferredoxin I gene from *Azotobacter vinelandii* using synthetic oligonuclotide probes. **J. Biosc.** 13: 323-327.

3. **Dimri, G. P.,** and H. K. Das (1988) Transcriptional regulation of nitrogen fixing genes by DNA supercoiling. **Mol. Gen. Genet.** 212: 360-363.

4. **Dimri, G. P.,** and H. K. Das (1990) Cloning and sequence analysis of *gyrA* gene of *Klebsiella pneumoniae.* **Nucl. Acids Res.** 18: 151-156.

5. **Dimri, G. P.,** d. Ari, L., Ames, G. -F. L. and J. C. Rabinowitz (1991) Physical mapping of *Escherichia coli* gene encoding the bifunctional enzyme 10-Methylentetrahydrofolate hydrogenase/ 5-10 Methenyl tetrahydrofolate cyclohydrolase. **J. Bacteriol.** 173: 5251.

6. **Dimri, G. P.,** Rudd, K. E., Morgan, M., Bayat, H. and G. -F. L. Ames (1992) Physical mapping of REP sequences in *Escherichia coli*, Phylogenetic distribution among *E.coli* strains and other enteric bacteria. **J. Bacteriol.** 174: 4583-4593.

7. **Dimri, G. P.**, and J. Campisi (1994) Altered profile of transcription factors binding activity during cellular senescence. **Exp. Cell Res.** 212: 132-140.

8. **Dimri, G. P.,** Hara, E. and J. Campisi (1994) Regulation of two E2F related genes in presenescent and senescent human fibroblasts. **J. Biol. Chem.** 269:16180-6186.

9. **Dimri, G. P.,** Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Rubelj, I., Pereira-Smith, O. M., Peacocke, M. and J. Campisi (1995) A Novel biomarker identifies senescent human cells in culture and in aging skin in vivo. **Proc. Natl. Acad. Sci. USA,** 92: 9363-9367.

10. Hara, E., Uzman, A., **Dimri, G. P.,** Nehlin, J., Testori, A. and J. Campisi (1996) The HLH protein ID1 complements an Rb binding deficient T antigen for stimulation of DNA synthesis in senescent human fibroblast. **Dev. Genet.** 18: 161-172.

11. **Dimri, G. P.,** Nakanishi, M., Desprez, P., Smith, J. R. and J. Campisi (1996) Inhibition of E2F activity by the cyclin dependent protein kinase inhibitor p21. **Mol. Cell. Biol**. 16: 2987-2997.

12. Good, G., **Dimri, G. P.,** Campisi, J. and K. Y. Chen (1996) Regulation of

dihydrofolate reductase and E2F genes in human diploid fibroblasts during senescence in culture. **J. Cell. Physiol.** 168: 580-588.

13. **Dimri,G. P.,** Itahana, K., Acosta, M. and J. Campisi (2000). Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14ARF tumor suppressor. **Mol. Cell. Biol.** 20: 273-285.

14. Itahana, K., **Dimri, G. P**. and J. Campisi (2001) Regulation of cellular senescence by p53. **Eur. J. Biochem.** 268: 2784-2791.

15. Li, B., Goyal, J., Dhar, S., **Dimri, G. P**., Evron, E., Sukulmar, S. and V. Band (2001) CpG methylation in exon 3 as a basis for breast tumor specific loss of NES1 expression. **Cancer Res**. 61: 8014-8021.

16. Itahana, K., **Dimri, G. P**. Itahana, Y., Zou, Y., Hara, E., Desprez, P. Y., and J. Campisi (2002) A role for p53 in maintaining and establishing quiescence growth arrest in human cells. **J. Biol. Chem.** 277: 18206-18214.

17. **Dimri, G. P**., Martinez, J. L., Jacobs, J. L, Keblusek, P., Itahana, K., van Lohuizen, M., Campisi, J. Wazer, D. E., and V. Band (2002) Bmi-1 oncogene induces telomerase and immortalizes human mammary epithelial cells. **Cancer Res.** 62: 4736-4745.

18. Kumar, A., Zhao, Y., Meng, G., Zeng, M., Srinivasan, S., Gao, Q., **Dimri, G.,** Weber, G., Wazer, D., Band, H., and V. Band (2002) Human papilloma virus oncoprotein E6 inactivates the transcriptional coactivator human ADA3. **Mol. Cell. Biol.** 22: 5801-5812.

19. Zeng, M., Kumar, A., Meng, G., Gao, Q., **Dimri, G.** , Wazer, D., Band, H., and V. Band (2002) HPV16 E6 oncoprotein inhibits RXR-mediated transactivation by targetting human ADA3 coactivator. **J. Biol. Chem.** 277: 45611-45618.

20. Itahana, K., Ying , Z., Itahana, Y, Martinez, J. L., Beausejour, C., Jacobs, J. L., van Lohuizen, M., Band, V., Campisi, J. and **G. P. Dimri** (2003) Control of replicative senescence in human fibroblast by p16 and the polycomb protein Bmi-1. **Mol. Cell. Biol.** 23: 389-401.

21. Meng, G., Zhao, Y., Nag, A., Zeng, M., **Dimri, G.**, Gao, Q., Wazer, D.E., Kumar, R., Band, H., and V. Band, (2004). Human ADA3 binds to estrogen receptor (ER) and functions as a coactivator for ER- mediated transactivation. **J. Biol. Chem.** 279:54230-5440.

22. Maurelli, R., Bondanza, S., Guerra, L., Abbruzzese, C., **Dimri, G.**, Gellini, M., Zambruno, G. and Dellambra, E. (2006). Inactivation of $p16^{Ink4a}$ immortalizes primary human keratinocytes by maintaining cells in the stem cell compartment.

FASEB J. In Press.

23. Song, L-B., Zeng, M.-S., Liao, W-T., Zhang, L., Mo, H-Y., Liu, W.-L., Shao, J-Y., Wu, Q-L., Li, M-Z., Xia, Y-H., Fu, L-W., Huang, W.-L., **Dimri, G**., Band, V. and Zeng, Y-X. (2006). Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizes primary human nasopharyngeal epithelial cells. **Cancer Res.** In Press.

24. Zhang, Y, J. Chen, C. B. Gurumurthy, Q. Gao, **G. Dimri**, D. Wazer, S. W. Lee, H. Band and V. Band. (2006). The human ortholog of Drosophila ecdysoneless protein interacts with p53 and regulates its function. **Cancer Res.** Accepted pending minor revision.

Manuscripts Submitted:

1. Kim, R. H., Z. M. Oo, S. J. Kim, Ki-H. Shin, **G. P. Dimri,** T. Han, R. Christensen, No-H. Park and Mo K. Kang (2006). Elevated expression of polycomb group protein Bmi-1 extends the replicative life span of normal human oral keratinocytes and is associated with head and neck cancer. Submitted.

2. Guo, Wei-Jian, S. Datta, M.-S. Zeng, L.-B. Song, B-H. Guo, P. Bommi, V. Band and **G. P. Dimri.** (2006) Mel-18, a polycomb group protein regulates senescence and oncogenesis via transcriptional repression of Bmi-1 and c-Myc oncoproteins. Submitted.

Manuscripts in Preparation

1. Datta, S., Guo, W.-J., Yadav, A., Dimri, M., Bommi, P., Band, H., Band, V. and **Dimri, G. P.** Bmi-1 oncoprotein cooperates with H-Ras to transform human mammary epithelial cells. In Preparation.

2. Yadav, A., Gandhari, M., Bommi, P., Band, V. and **Dimri, G. P.** Genotoxic drugs induce Bmi-1 degradation in a phosphorylation-dependent manner via proteosome-mediated pathway. In Preparation.

3. Yadav, A., Datta, S., Guo, W.-J., Bommi, P., Band, V. and **Dimri, G.P.** Bmi-1 interacts with mdm2 and regulates its expression in mammary epithelial cells. In Preparation.

Peer Reviewed Reviews & Commentaries:

1. Campisi, J., **Dimri, G. P.,** Nehlin, J. O., Testori, A. and K. Yoshimoto (1996) Coming of age in culture. **Exp. Geron.** 31: 7-12.

2. **Dimri, G. P.,** Testori, A. and J. Campisi (1996) Replicative senescence, aging and growth regulatory transcription factors. **BioSig.** 5:154-162.

3. Itahana, K., Campisi, J. and **G. P**. **Dimri** (2004) Mechanisms of cellular senescence in human and mouse cells. **Biogeron.** 5: 1-10.

4. **Dimri G. P.** (2004) The search for biomarkers of aging: next stop INK4a/ARF locus. **Sci Aging Knowledge Environ. (**On-line sister publication of **Science)** Nov 03;2004(44):pe40.

5. **Dimri, G. P.**, Band, H and V. Band (2005) Mammary epithelial cell transformation: insights from cell culture and mouse models. **Breast Cancer Res.** 7: 171-179.

6. **Dimri, G.P.** (2005) What has senescence got to do with cancer? **Cancer Cell** 7: 505-512.

Proceedings, Book Chapters and Non-Refereed Papers:

1. Reddy, A. N., Phadns, S. H., **Dimri, G. P.,** Jaferi, S., Medhora, M. M., and H. K. Das (1986) Complexity of the Genome of *Azotobacter vinelandii.* In Biotechnology in Agriculture Eds Natesh, S., Chopra V. L., and S. Ramachandran, pp 15-19; Oxford & IBH Publishing Co. Pvt. LTD., New Delhi.

2. **Dimri, G. P.,** and J. Campisi (1995) Molecular and cell biology of replicative senescence. In Cold Spring Harbor Laboratory Symposium in Quantitative Biology : **Molecular Genetics of Cancer,** 54: 67-73.

3. Campisi, J. **Dimri, G. P.** and E. Hara (1996) Control of replicative senescence. In **Handbook of Biology of Aging,** 4th ed., Eds Schneider, E. and J. W., Rowe, pp121-149.

4. Itahana, K., Campisi, J. and **G. P**. **Dimri.** (2006) Methods to detect biomarkers of cellular senescence: the senescence associated β-galactosidase. **Methods in Molecular Biology volume on "Biological Aging: Methods and Protocol",** The Humana Press Inc., New Jersey, USA. In Press

Abstracts:

1. **Dimri, G. P.** and J. Campisi (1994) Transcriptional control of cellular replicative senescence. Molecular Biology of the Cell. 5, 386 a.

2. Danahy, J. F., Lee, X., Scott, G., **Dimri, G. P.** , Campisi., J. and M. Peacocke (1994) A biomarker of human cellular aging in vivo and in vitro. AFCR, Clinical Research Meeting, April 29-May 2.

3. **Dimri, G. P**., Hara, E., Acosta, M., Desprez, P., Nakanishi, M., Smith, J. R. and

J. Campisi (1995) The role of p53 in cellular replicative senescence. In FASEB J., 9, Abs # 1202.

4. **Dimri, G. P**., Acosta, M. and J. Campisi (1996) Regulation of E2F related genes during cellular senescence. Molecular Biology of the Cell. 9, Abs# 3102.

5. **Dimri, G. P.** and J. Campisi (1997) Extension of replicative life span and induction of crisis in E6 expressing fibroblasts by E2F1 overexpression. In Molecular and Genetic Strategies for Treatment of Age-Related Diseases. NMHCC Conference. July 15-16 Seattle, WA.

6. **Dimri, G. P.** and J. Campisi (1998) Extension of replicative life span and induction of crisis in E6 expressing fibroblasts by overexpression of E2F1. In FASEB J., 12: No. 4, Abs. #1846.

7. Krtolica, A., Yip, D., **Dimri, G. P**., Desprez, P. and J. Campisi (2000) The double-edged sword of replicative senescence: Senescent fibroblasts stimulate pre-malignant epithelial cell growth. AACR 91st Annual Meeting April 1-5, San Francisco, CA.

8. Li, B., Goyal., J., Dhar, S., **Dimri, G.,** Evron, E., Sukumar, S., Waer, D. E., and V. Band (2002) CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. Era of Hope Meeting, Abs# P8

9. Datta, S., Band, V. and **Dimri, G. P.** (2005) Polycomb proteins and Breast Epithelial cell transformation. Era of Hope, Dept. of Defense, Breast Cancer Research Program Meeting. Abst # P11-9.

10. Yadav, A., Datta, S., Band, V. and **Dimri, G. P.** (2005) Role of p53 in Mammary Epithelial Cell Senescence. Era of Hope, Dept. of Defense, Breast Cancer Research Program Meeting. Abst # P27-8.

Invited Presentations:

1994:	Biology of Aging, Special interest subgroup meeting, American Society for Cell Biology, Thirty Fourth Annual Meeting, San Francisco, CA
1998:	Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, IL., " Cellular and Molecular Biology of senescence"
1999:	California Pacific Medical Center, San Francisco, CA "Role of Cellular Senescence in Aging and Cancer"
1999:	Center for Aging, University of Alabama at Birmingham, AL "Role of Senescence in Aging and Cancer "
1999:	New England Medical Center, Boston, MA

	"Mechanism of Cellular Senescence in Human Cells"
2003:	8th World Congress on Advances in Oncology and 6th International Symposium on Molecular Medicine 16-18th October, 2003, Creta Maris, Hersonissos, Crete, Greece "Molecular Mechanisms of Cellular Senescence in Human Cells".
2005:	"What has senescence got to do with cancer?" Children's Memorial Research Center, Northwestern University, Chicago, IL "The role of Bmi-1 and Bmi-1 related genes in Senescence and Proliferation" Gheens Center for Research in Aging,University of Louisville, School of Medicine, Louisville, KY
2006:	"The role of Bmi-1 and Bmi-1-related Polycombs in Senescence and Oncogenesis" National Cancer Institute, NIH, Bethesda, MD

Mel-18, a polycomb group protein regulates cell proliferation, senescence and oncogenesis via transcriptional repression of Bmi-1 and c-Myc oncoproteins

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ABSTRACT:

Polycomb group (PcG) protein Bmi-1 is an important regulator of oncogenesis and stem cell-ness. It regulates senescence and proliferation of cells via transcriptional repression of INK4a/ARF locus and other target genes. Here, we report that Mel-18, a PcG ring finger protein (PCGF) transcriptionally regulates Bmi-1. Furthermore, the expression of Bmi-1 and Mel-18 inversely correlated in proliferating and senescent human cells and in a significant number of breast tumor samples. Bmi-1 downregulation by Mel-18 resulted in accelerated senescence and shortening of the replicative life span in normal cells and reduction of the transformed phenotype in malignant cells. Using promoter-reporter and chromatin immunoprecipitation (ChIP) assays and RNA interference (RNAi) approach, we also demonstrate that Bmi-1 is a bona fide target of c-Myc oncoprotein. Finally, our data suggest that Mel-18 regulates Bmi-1 expression via downregulation of c-Myc. Thus, our studies link c-Myc and polycomb function in senescence, cell proliferation and oncogenesis.

INTRODUCTION

Polycomb group (PcG) proteins are chromatin-modifying proteins, which play an important role in the development (38). Besides a role in the development, these proteins also regulate tumorigenesis (15, 43). In particular, EZH2 and Bmi-1 overexpression has been linked to invasive breast and prostate cancers (14, 25, 26, 45). Bmi-1 is also overexpressed in mantle cell lymphoma (5), B-cell non-Hodgkin lymphoma (44), myeloid leukemia (40), non-small cell lung cancer (46), colorectal cancer (27), hepatocellular carcinoma (34), pediatric brain tumors (17) and medulloblastomas (29). In addition to its role in oncogenesis, recent work from several laboratories indicates that Bmi-1 is required for self-renewal of hematopoitic stem cells (HSCs) and neural stem cells in murine models (21, 28, 33, 36). Bmi-1 is also possibly involved in the maintenance and proliferation of breast stem cells (30).

After a finite number of cell divisions, most normal human cells undergo cellular senescence, whereby cells cease to divide (8, 10). Senescence constitutes a tumor suppressor mechanism (8, 10) and bypass of senescence is required for tumorigenesis (10). The exact role of PcG proteins in tumorogenesis is still unclear. However, some of the polycomb proteins, such as Bmi-1 and EZH2, are known to regulate senescence and proliferation via well-known growth regulatory pathways (6, 20, 22). For example, Bmi-1 negatively regulates INK4a/ARF locus (22), which may impact both p16-pRb and ARF-p53-p21 pathways of cellular senescence (10). Indeed, Bmi-1 has been shown to regulate cellular senescence in murine and human cells (20, 22). Bmi-1 is also thought to prevent premature senescence of neural stem cells by repressing INK4a/ARF locus (7, 32).

Premature senescence of cells may also contribute to organismic aging (8). If so, the regulators of senescence are likely to play a role in aging. Indeed, downregulation of Bmi-1 by the disruption of the SNF2-like gene PASG was shown to result in growth retardation and premature aging in a murine model (41).

Bmi-1 is a particularly interesting oncoprotein; it not only regulates the INK4a/ARF locus, but can also immortalize human mammary epithelial cells (HMECs) (13). We recently reported that Bmi-1 expression is downregulated during replicative senescence (20). Besides senescence-specific regulation, molecular pathways that regulate Bmi-1 expression are unknown. Identification of such regulatory pathways is important for our understanding of the role of Bmi-1 and other PcG proteins in oncogenesis, stem cell biology and aging.

In addition to Bmi-1, mammalian cells also express Mel-18 (also known as polycomb group ring finger 2 (PCGF2)), a closely related PcG protein (19). The Mel-18 gene product is structurally highly similar to Bmi-1. Its N-terminal region, which contains a RING finger domain, is 93% homologues to Bmi-1 (19). The homology towards the C-terminal region, which contains a nuclear localization signal and a proline-serine rich (PS) domain, is less conspicuous than the N-terminal region (19). Bmi-1 and Mel-18 are known to interact and thought to be the constituents of PRC1 (Polycomb Repressive Complex 1) (4, 38). However, a recent study suggests that Mel-18 may not be part of PRC1 (9), although it could structurally but not functionally replace Bmi-1 in the PRC1 complex (9).

It is thought that Bmi-1 and Mel-18 regulate overlapping and unique sets of genes (3, 24, 42). However, unlike Bmi-1, Mel-18 binds to a well-defined nucleotide sequence '5- GACTNGACT-3' present in the promoter region of its target genes (24). One of the unique target gene of Mel-18 is c-Myc, which is transcrpitionally repressed by Mel-18 (42). The exact role of Mel-18 in senescence, proliferation and oncogenesis is unclear. Although its structural similarities to Bmi-1 suggest it to be an oncoprotein, few studies indicate that Mel-18 in fact may be a tumor suppressor (24, 31, 42) and that it may negatively regulate self-renewal of HSCs (23). Despite high similarity between Bmi-1 and Mel-18, we found that Mel-18 functions as a transcriptional repressor of Bmi-1 expression. We also report that Bmi-1 promoter region contains a functional c-Myc binding site through which c-Myc regulates expression of Bmi-1. Because Mel-18 downregulates c-Myc expression and Bmi-1 is a c-Myc target, our data suggest that Mel-18 regulates expression of Bmi-1 via repression of c-Myc.

MATERIALS AND METHODS

Cellular reagents and methods. WI-38 and BJ fibroblasts were obtained from J. Campisi (Lawrence Berkeley National Laboratory). The MRC-5 fibroblast strain was obtained from the NIA Aging Cell Repository, Coriell Institute for Medical Research, Camden, NJ). The fibroblasts strains were grown and serially passaged, and the onset of senescence in fibroblasts was determined using SA-β-gal assay as described (12, 20). MCF10A and MCF7 cells were cultured as described (13). Stable cell lines expressing Mel-18 or other gene of interest were generated by infection of the retroviral vectors expressing the particular gene as described (11). The retroviruses were produced by transient transfection

of the retroviral vector together with pIK packaging plasmid into tsa 54 packaging cell line as described (11). Soft-agar assays to determine the anchorage independence of cells were done as described (25).

Retroviral expression and shRNA vectors. The vectors containing cDNAs of Mel-18, NSPC1 and MBLR and c-Myc were obtained from ATCC (American Type Culture Collection, Manssas, VA). The individual cDNAs were amplified and cloned either in pLPC retroviral vector obtained from Dr. J. Campisi (originally from Dr. T. deLange, Rockefeller University, NY) or in pBabe-puro (11). Bmi-1 and Mel-18 sh RNAs were designed and cloned in the retroviral vector pRS (retro-super) obtained from Oligoengine Inc. (Seattle, WA). The sequences of shRNA were- Mel-18- #1: CGACGCCACCACUAUCGUG ; #2: AGACCAACAAAUACUGCC C; and Bmi-1 shRNA- #1 GUUCACAAGACCAGACCAC and
#2 GACCAGACCACUACUGAAU. Additional shRNAs targeting Mel-18 and c-Myc were obtained from Open Biosystems (Huntsville, AL).

Promoter-reporter vectors and lucifearse assays. The promoter region of Bmi-1 was identified by BLAST comparison of the untranslated region of Bmi-1 cDNA with human genomic clones and analyzing region further upstream of it. The putative promoter region was amplified using a BAC clone (RP11-573G6 obtained from Children's Hospital Oakland Research Institute, Oakland, CA), and cloned into the pGL3 luciferase reporter vector (Promega Corporation, Madison, WI). Similarly, the mutant promoter fragments

were generated by PCR, and cloned into the pGL3 vector. The reporter assays were done as described [29] using a luciferase assay kit from Promega Corporation.

ChIP assays. Chromatin immunoprecipitation linked PCR (ChIP) assay was performed using a kit from Upstate Cell Signalling Solutions (Charlottesville, Virginia). Briefly, c-Myc cross-linked to its binding sites was immunoprecipitated using A14 rabbit polyclonal antibody against c-Myc (Santa Cruz Biotech (Santa Cruz, CA). The immunoprecipitated chromatin was amplified using 5'ACGGGCCTGACTACACCGACACT3' and 5'CTGAAGGCAGAGTGGAAACTGACAC3' primers, which flank c-Myc binding site of the Bmi-1 promoter. The primers- 5'TTCAAAGGCATCTTCTGCAG3' and 5'CTTAACCGCCCAGATACATC3', which amplify a non-Myc binding region were used as a control.

RT-PCR assays. The RT PCR was carried out as described (13). Briefly, cDNA was generated using a one step PCR kit from Gibco-BRL. The PCR primers to detect Bmi-1 expression in control and Mel-18 overexpressing cells were- 5'CATGTATGAGGAGGAACC3'; 5'AACTGTGGATGAGGAGAC3'

Immunological reagents and methods. Bmi-1 was detected using either F6 mouse monoclonal antibody (mab) from Upstate Cell Signaling Solutions (Charlottesville, Virginia), or 1H6B10G7 mab from Zymed (S. San Francisco, CA). Mel-18 was detected by a rabbit polyclonal H-115 (Santa Cruz Biotech., CA). The 9E10 mab (Santa Cruz Biotech., CA) against c-Myc was used to detect the expression of c-Myc tag in

exogenously expressed proteins. Western blot analyses to detect the expression of Bmi-1, Mel-18, c-Myc were performed as described (13, 20).

Clinical samples, immunohistochemical and statistical analysis. A total of 61 breast cancer tissue samples were collected from the archives of the Department of Pathology, Cancer Center, Sun Yat-sen University (Guangzhou, China). For the use of these clinical materials for research purposes, prior patients' consent and approval from the Institute Research Ethics Committee was obtained. Bmi-1 and Mel -18 were detected using antibodies described above.

Briefly, the paraffin sections of the breast cancer tissue from the patient were deparaffinized with xylene and rehydrated. Antigenic retrieval was processed by submerging the sample in citrate buffer (pH 6.0) and microwaving. The sections were then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 1% BSA to block the non-specific binding. The sections were then stained with anti-Bmi-1 antibody (1:100) or anti-Mel -18 antibody (1:200) at 4°C overnight. After washing, the tissue sections were then incubated with the biotinylated anti-mouse or anti-rabbit secondary antibody, followed by further incubation with streptavidin-horseradish-peroxidase complex. The tissue section was immersed in 3-amino-9-ethyl carbazole (AEC), and counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted in crystal mount. In the negative control, primary antibody was replaced by the non-immune mouse IgG of the same isotype.

All slides were interpreted by two independent observers in a blinded fashion. For each case, at least 1,000 tumor cells were analyzed and the percentage of nuclear stained tumor cells was recorded. For each sample, one score was given according to the percent of positive cells as <5% of the cells: 1 point, 6-35% of the cells: 2 point, 36-70% of the cells: 3 point, >71% of the cells: 4 point, another score was given according to the intensity of staining as negative staining: 1 point, weak staining: 2 point, moderate staining: 3 point, and strong staining: 4 point. A final score was then calculated by multiplying the above two scores. If the final score was equal or bigger than four, the tumor was considered positive; otherwise, the tumor was considered negative. All statistical analyses were done by using the SPSS 10.0 software package. Spearman's rank correlation was used to estimate the correlation between Bmi-1 and Mel-18 expression.

RESULTS

Mel-18 regulates Bmi-1 expression. To gain further insight into the polycomb biology, we cloned cDNAs of Bmi-1 related PcG proteins into a retroviral expression vector pLPC1. Using this vector, we stably overexpressed Mel-18 (19), NSPC1 (Nervous System Polycomb-1), also called PCGF1 (35) and MBLR (Mel18 and Bmi1-like RING finger protein) (2) in MCF10A cells (Fig. 1A left panel). We also overexpressed Bmi-1 using pBabe-Bmi-1 (13) in these cells. Our results indicated that Mel-18 but not NSPC1 or MBLR downregulates endogenous Bmi-1 (Fig. 1A right panel, compare lanes 1 and 4). Similar results were obtained when Mel-18 was stably overexpressed in the MCF7 breast cancer cell line and MRC-5 fibroblasts (Fig. 1B). We also examined the expression of c-

Myc in Mel-18 overexpressing cells. Consistent with the published report (42), Mel-18 overexpression led to c-Myc downregulation (Fig. 1B).

To rule out the possibility of unknown genetic changes contributing to Bmi-1 downregulation during selection of the stable expression of Mel-18, we also performed transient transfection assays in 293T cells. Increasing concentrations of transiently transfected Mel-18 resulted in a corresponding downregulation of endogenous Bmi-1 and c-Myc in these cells (Fig. 1C). The regulation of Bmi-1 by Mel-18 was further confirmed by the RNA interference (RNAi) approach. MCF10A and MRC-5 cells expressing Mel-18 short inhibitory RNA (shRNA) were generated. Two Mel-18 shRNA constructs were used; both downregulated Mel-18 in MCF10A (Fig. 1D) and MRC-5 (Fig. 1E) cells. However, downregulation of Mel-18 was less conspicuous in MCF10A cells. In agreement with the above data, stable expression of both Mel-18 shRNAs upregulated Bmi-1 expression in MRC-5 and MCF10A cells (Fig. 1E). As expected, knockdown of Mel-18 also upregulated c-Myc expression (Fig. 1D). These results suggest that Bmi-1 and c-Myc are physiological targets of Mel-18.

RING finger of Mel-18 is required for the downregulation of Bmi-1. To identify the structural domain(s) of Mel-18 required for Bmi-1 downregulation, we generated ΔRF (lacks RING finger domain), ΔRFNLS (lacks RING finger and nuclear localization signal) and ΔPS (lacks proline-serine rich region) mutants of it (Fig. 2A). These mutants were either stably overexpressed in MCF10A cells (Fig. 2B) or transiently overexpressed in 293T cells (Fig. 2C). The results indicated that wild type Mel-18 and the ΔPS mutant of it,

both contained intact RING finger domain downregulated Bmi-1 expression suggesting that the RING finger domain of Mel-18 is required for downregulation of Bmi-1. Interestingly, ΔRF and ΔRFNLS mutants of Mel-18 upregulated Bmi-1, suggesting potential dominant negative (DN) activity of these mutants (Fig. 2B and 2C).

Mel-18 transcriptionally downregulates Bmi-1 gene expression. We next determined the mechanism of downregulation of Bmi-1 by Mel-18. Because, Mel-18 contains a RING finger domain and RING finger proteins can function as E3 ubiquitin ligase and promote protein degradation via proteosome pathway (37), we hypothesized that Mel-18 may downregulate Bmi-1 at the protein level. To examine this possibility, we subjected Mel-18 overexpressing and control cells to treatment with proteosome inhibitor MG-132 and determined Bmi-1 protein level by western blot analysis. The results indicated that MG-132 treatment did not significantly increase Bmi-1 protein level, suggesting that Mel-18 does not regulate Bmi-1 by promoting its degradation via proteosome pathway (Fig. 3A). To further confirm this result, we determined the half-life of Bmi-1 protein in control and Mel-18 overexpressing cells using cyclohexamide treatment (Fig. 3B). We did not find any significant difference in the half-life of Bmi-1 in control, and Mel-18 overexpressing cells indicating that Bmi-1 is not regulated at the protein level by Mel-18 (Fig. 3B).

Since, Mel-18 did not appear to regulate Bmi-1 expression via protein stability, we surmised whether Mel-18 could regulate the transcription of *Bmi-1* gene. To examine this possibility, we first performed a semi-quantitative RT-PCR to determine the mRNA level of Bmi-1 in control and Mel-18 overexpressing cells. Our data indicated that Mel-18

downregulates mRNA levels of Bmi-1 (Fig. 3C). This result suggests that Mel-18 possibly regulates Bmi-1 transcription.

c-Myc regulates Bmi-1 transcription via the c-Myc binding site present in its promoter. To examine the possibility of Mel-18 regulating Bmi-1 transcription, we analyzed 400 bp of Bmi-1 5' untranslated region (UTR) containing *Bmi-1* promoter. The analysis of binding sites for various transcription factors was done using TFSEARCH version 1.3. This analysis showed that the Bmi-1 promoter is a GC rich promoter without a well-defined TATA sequence, and that it contains numerous potential SP-1 binding sites (Fig. 4A). We did not find any potential binding sites (GACTNGACT) for Mel-18. However, the sequence analysis showed the presence of a perfect E-box sequence (CACGTG), which is a potential binding site for Myc family of transcription factors (1). The importance of Myc binding sites in the Bmi-1 promoter was further underscored by the fact that this site is also present in the mouse Bmi-1 promoter (data not shown).

To determine if indeed Bmi-1 is regulated by c-Myc via E-box present in the Bmi-1 promoter, we first performed chromatin immunoprecipitation linked PCR (ChIP) assay using vector control and Mel-18 overexpressing MCF7 and MCF10A cells. The cross-linked chromatin was immunoprecipitated (IPed) using a rabbit polyclonal antibody (ab) against c-Myc and the control rabbit IgG, and the PCR was performed using primers (c-Myc primer set) that flank c-Myc binding sites (E-box) in the Bmi-1 promoter. A primer set derived from further upstream sequences that does not flank the c-Myc binding site was used as a control primer set. The results indicated that c-Myc primer set was specifically

able to amplify the PCR product of an expected size (200 bp) from the vector control cells (Fig. 4B). The yield of the PCR product was much less in Mel-18 overexpressing cells indicating the downregulation of c-Myc in Mel-18 overexpressing cells (Fig. 4B). The control primer set using c-Myc and IgG IPed extracts did not yield any PCR product indicating the specificity of binding of c-Myc to E -box present in the Bmi-1 promoter (Fig. 4B). We further cloned the E-Box region (150 bp) of the Bmi-1 promoter in the pLuc vector (pLuc-Myc), which contains a minimal promoter and studied c-Myc regulation of the reconstituted promoter. The results strongly indicated that the E-box presence in Bmi-1 promoter is functional. Transient co-transfection of c-Myc increased the activity of the reconstituted promoter (Fig. 4C).

We utilized a luciferase reporter vector pGL-3 to generate three different Bmi-1 promoter-reporter constructs (Fig. 4D). pGL3-Bmi PrWT contained +45 to -233 region of Bmi-1 promoter and untranslated region of Bmi-1 mRNA; in pGL3-Bmi PrMut, the Myc binding site (E-box) was mutated from CACGTG to CGCGTG. The third construct pGL3-Bmi PrΔMyc contained a deletion of c-Myc binding site. We determined the luciferase activity driven by wild type or mutant promoters. The results indicated that wild type promoter display robust promoter activity while the mutant promoters exhibited 50% less activity compared to the wild type promoter (Fig. 4D). We further studied the regulation of Bmi-1 promoter by c-Myc and Mel-18 (Fig. 4E, 4F and 4G). The promoter-reporter constructs were cotransfected with increasing amounts of Mel-18 overexpressing plasmid (Fig. 4E), c-Myc overexpressing plasmid (Fig. 4F) or a plasmid expressing c-Myc shRNA (Fig. 4G). Analysis of the luciferase activity of these promoter-reporter constructs

suggested that Mel-18 negatively regulates Bmi-1 promoter through the c-Myc binding site, as the promoter that lacked E-box or contained mutant c-Myc binding site did not respond to increasing concentrations of the Mel-18 expressing plasmid (Fig. 4E). Furthermore, the transient cotransfection of c-Myc overexpressing plasmid led to the upregulation of activity of wild type but not mutant promoters (Fig. 4F). Similarly, knockdown of c-Myc expression by transfection of a plasmid expressing c-Myc shRNA downregulated Bmi-1 promoter activity of the promoter that contained wild type c-Myc binding site (Fig. 4G).

Bmi-1 is a bona fide target of c-Myc, and c-Myc overexpression rescues Mel-18 mediated repression of Bmi-1 expression. Our promoter-reporter analysis suggested that c-Myc positively regulates the expression of Bmi-1. To further confirm that endogenous promoter of Bmi-1 is regulated by c-Myc, we studied the expression of Bmi-1 in MCF10A cells, which stably overexpress c-Myc under a retroviral promoter (Fig. 5A), and MCF10A cells where the expression of c-Myc was stably knockdown by the RNAi approach (Fig. 5B). Our data suggest that the stable overexpression of c-Myc results in constitutively high Bmi-1 expression (Fig. 5A). Accordingly, we also found that knockdown of c-Myc expression using RNAi approach, particularly Myc RNAi #2, which was very effective in c-Myc knockdown, results in a substantial downregulation of endogenous Bmi-1 expression (Fig. 5B).

We next carried out a c-Myc rescue experiment. Since Mel-18 represses c-Myc expression by binding to its native promoter, we reasoned that c-Myc overexpression using

a heterologous promoter should rescue Bmi-1 repression caused by Mel-18 overexpression. To test this hypothesis, we transiently transfected Mel-18 together with pCMV-Myc. Our results indicated that indeed c-Myc overexpression using CMV promoter rescues Mel-18 mediated repression of endogenous Bmi-1 (Fig. 5C). Collectively, our data strongly suggest that Mel-18 downregulate Bmi-1 expression at the transcription level through c-Myc binding site and that c-Myc acts as a positive regulator of Bmi-1 expression.

Mel-18 expression is upregulated during replicative senescence in human cells.

We have previously reported that Bmi-1 expression is downregulated during replicative senescence in human cells (20). The molecular basis of Bmi-1 downregulation during senescence is unknown. Based on our data, we surmised that Mel-18 expression might be upregulated during senescence, which would result in downregulation of Bmi-1 expression. Conversely, low levels of Mel-18 or absence of its expression in presenescent cells may permit high Bmi-1 expression in these cells. To examine this possibility, we prepared total cell extract from presenescent, or young (Y), and senescent, or old (O), cells of MRC5 and BJ fibroblast strains and determined Mel-18, Bmi-1 and p16 expression by western blot analysis. The onset of senescence in these fibroblast strains was determined using senescence associated β-galactosidase (SA-β-gal) marker (12). Consistent with our previous results (Itahana et al. 2003), Bmi-1 was downregulated during senescence (Fig. 6A). Our results also indicated that p16 was conspicuously upregulated in MRC-5 fibroblasts, but not in senescent BJ fibroblast, which expressed much lower levels of p16 even during senescence (20).

Our data indicate that indeed Mel-18 expression is virtually undetectable in presenescent (Y) cells and is upregulated in senescent (O) fibroblasts (Fig. 6A). Consistent with previously published literature, senescent cells contained high levels of underphosphorylated pRb (Fig. 6A). Upregulation of Mel-18 in senescent cells could result due to the growth arrest stage of these cells and not necessarily be due to senescence. To rule out this possibility, we also examined Mel-18 expression in quiescent cells, which are growth arrested by serum starvation. The results indicated that growth arrest due to quiescence do not increase Mel-18 expression suggesting that the upregulation of Mel-18 is senescence-specific and not due growth arrest per se (Fig. 6B). Consistent with our earlier data, Bmi-1 was downregulated in senescent (O) but not in quiescent cells (YQ). Importantly, Bmi-1 expression was inversely correlated with Mel-18 expression during all three growth conditions - senescence (O), quiescence (YQ), and proliferation (YGr).

Mel-18 overexpression leads to accelerated cellular senescence and shortening of the replicative life span. Upregulation of Mel-18 during senescence suggests that it may contribute to senescence or be a causative factor for senescence via downregulation of Bmi-1 and/or other important undefined target genes. To examine this possibility, we overexpressed Mel-18 in presenescent MRC-5 fibroblasts and determine the onset of senescence in control and Mel-18 overexpressing cells (Fig. 6C). Our results indicated that indeed, Mel-18 overexpression accelerates entry of cells into senescence, as determined by SA-β-gal marker, and shortens replicative life span (Fig. 6C). We also used a stable expression of shRNA against Bmi-1 and determined the replicative life span of MRC-5

fibroblasts. The results indicated that similar to Mel-18 overexpression, Bmi-1 knockdown by RNAi approach accelerates entry of cells into senescence (Fig. 6D). We further determined if knockdown of Mel-18 expression extends the replicative life span, as the Mel-18 downregulation results in upregulation of Bmi-1, which is known to extend the replicative life span in human cells (Itahana et al. 2003). Indeed, stable expression of Mel-18 shRNA extended the replicative life span in MRC-5 fibroblasts. Mel-18 shRNA expressing cells also exhibited considerably less numbers of senescent cells at a given time point (Fig. 6E).

To determine the mechanism of senescence induction by Mel-18, we examined the expression of various regulators of senescence (10) in control and Mel-18 overexpressing cells. Western blot analysis of Bmi-1, pRb and p16 suggested that Mel-18 overexpression led to p16 upregulation, Bmi-1 downregulation, and pRb hypophosphorylation, suggesting that Mel-18 regulates the p16-pRb pathway of senescence (Fig. 6F). Similarly, knockdown of Bmi-1 expression upregulated p16 and resulted in accumulation of underphosphorylated pRb (Fig. 6F). We did not find any significant alterations in expression of p53 in Mel-18 overexpressing cells.

Overexpression of Mel-18 reduces malignancy of breast cancer cells. Tumor suppressors regulate senescence and often overexpression of tumor suppressors can induce senescent phenotype (10). Expression of senescence causing genes may reduce or even revert tumorigenic phenotypes of malignant cells. To examine this possibility, we determined the transformation potential of control and Mel-18 overexpressing MCF7 cells

using anchorage-independence growth assay. The results indicated that Mel-18 overexpression in MCF7 cells led to a decrease in colony formation in soft agar (Fig. 7A). The colonies in Mel-18 overexpressing MCF7 cells were less in frequency and smaller in size (Fig. 7A upper panel). We also determined the colony formation potential of MCF7 cells, which expresses Bmi-1 shRNA. Our results indicated that similar to Mel-18 overexpression Bmi-1 downregulation by RNAi, this approach led to significant decreases in transformed phenotype of MCF7 cells (Fig. 7A lower panel). Western blot analysis confirmed overexpression of Mel-18 and downregulation of Bmi-1 in these cells (Fig. 7A upper panel). The data also confirmed that Bmi-1 shRNA#1 was not effective in downregulation of Bmi-1 (Fig. 7A lower panel) and did not result in significant decrease in colony formation (Fig. 7A lower panel).

Bmi-1 and Mel-18 expression inversely correlates in breast tumors. Our data in cultured human fibroblasts and mammary epithelial cells suggest an inverse correlation between Bmi-1 and Mel-18 expression. Many breast tumors overexpress Bmi-1 (14, 26). We hypothesize that Mel-18 downregulation may lead to Bmi-1 upregulation in breast tumors. To examine this possibility, we studied the expression of Mel-18 and Bmi-1 in 61 breast tumors by immunohistochemistry (Fig. 7B). By immunohistochemical analysis, 51 of 61 (83.6%) paraffin-embedded archival breast tumor biopsies showed a positive staining for Bmi-1, while 15 of 61(24.5%) of the biopsies showed a positive staining of Mel 18. Of 15 Mel-18 and 51 Bmi-1 positive biopsies, only 6 were positive for both Bmi-1 and Mel-18. The correlation between Bmi-1 and Mel 18 expression was further analyzed by

Spearman correlation analysis, which showed a strong negative correlation (r=-0.673, P=0.000).

DISCUSSION

Various PcG proteins form higher order complexes such as PRC1 and PRC2 in cells (38). These complexes are thought to regulate expression of target genes such as members of the Hox family (38). When over- or under-expressed, individual polycomb proteins such as Bmi-1 can also regulate expression of its target genes. Virtually nothing is known about the regulation of the expression of various PcG proteins. Here, we report a novel observation that Bmi-1 is specifically regulated by another PcG protein Mel-18, but not by other related polycomb proteins such as MBLR and NSPC1.

Our novel observation suggests that Mel-18 is an upstream negative regulator of Bmi-1 function, which promotes oncogenesis and stem cell-ness. Consistent with such an observation, Bmi-1 knockdown by RNAi as well as its downregulation by Mel-18 overexpression in cells resulted in decrease in colony formation suggesting a tumor suppressor role for Mel-18. Moreover, Mel-18 expression is upregulated in senescent cells. As senescence constitutes a tumor suppressor mechanism and is regulated by tumor suppressors (10), our results clearly place Mel-18 in the tumor suppressor category. It is known that various tumor suppressors are either upregulated (for example p16) or the physiological activity of tumor suppressors is upregulated during senescence (10). For example, DNA binding activity of p53 is upregulated during senescence and there is a relative increase in underphosphoryalted pRb as compared to hyperphosphorylated pRb

(10). Forced expression of p16, p14ARF and other tumor suppressors has been shown to accelerate senescence in human cells (10). Consistent with these properties of tumor suppressors, we found that Mel-18 is upregulated during senescence in human fibroblasts.

Further support for the function of Mel-18 as a tumor suppressor is suggested by the observation that the forced expression of Mel-18 induces accelerated senescence in human fibroblasts. Accelerated senescence in Mel-18 overexpressing fibroblasts is accompanied by increase in pRb hypophosphorylation, p16 upregulation and downregulation of Bmi-1 in MRC-5 fibroblasts. On the other hand, in BJ fibroblasts, which express very low levels of p16, no upregulation of p16 was observed. However, Bmi-1 downregulation and increase in pRb hypophosphorylation was evident, suggesting that Bmi-1 may regulate pRb phosphorylation independent of p16, possibly by other CDK inhibitors. We are currently studying the regulation of other CDK inhibitors by Mel-18 and Bmi-1.

In contrast to wild type Mel-18, RING finger mutants of it upregulate Bmi-1. We speculate that these mutants may upregulate Bmi-1 by inhibiting function of endogenous Mel-18, by binding and sequestering it in the cytoplasm and thus exhibit dominant negative (DN) activity. Alternatively, RING finger mutants may bind to the promoter of presumptive target(s), which may be Bmi-1 itself or the other target(s) that regulate Bmi-1. However, if this was the case, ΔRFNLS should not have exhibited a DN activity because it lacks the nuclear localization signal. Although, Mel-18 is thought to directly bind to DNA in a sequence specific manner (42), at present the DNA binding domain of Mel-18 is

unknown. Since RING finger can bind to DNA, it is possible that Mel-18 binds to its target sequences via RING domain. In this scenario, RING mutants of Mel-18 also can function as DN mutants. Detail characterization of DNA binding domain and possible DN activity of RING mutants remains to be examined.

Although, Mel-18 regulates its target genes by binding to the promoter region, Bmi-1 promoter does not contain presumptive Mel-18 binding sequences. However, it remains possible that Mel-18 regulate Bmi-1 expression by repressing a positive regulator of Bmi-1. Indeed, we found that Bmi-1 promoter contains an E-box to which a positive or negative regulator of Bmi-1 can bind. Identification of an E-box in the Bmi-1 promoter is very intriguing from an oncogenesis point of view. A number of E-box binding proteins are known, some of which act as repressors, while others act as activators of transcription. The c-Myc family of transcription factors, which bind to E-box, are clearly implicated in oncogenesis. The c-Myc oncogene is amplified and/or overexpressed in a variety of malignancies (see Myc Cancer Gene web site: http://www.myccancergene.org). It acts as a transcription factor and regulates expression of a number of genes (1, 47). However, it is still unclear what the cancer relevant bona fide targets of c-Myc are. Here, we identified Bmi-1 oncogene as an important target of c-Myc oncoprotein. Similar to our results, a very recent report has also implicated c-Myc in regulation of Bmi-1 expression (16).

c-Myc oncoprotein dimerizes with Max, which is usually in excess. Myc-Max complexes positively regulate expression of Myc target genes. Myc and Max also dimerizes with Mad1, Mxi-1, Mad3, Mad4 and Mnt (1). Heterodimers of these proteins

also bind to E-box and often negatively regulate the expression of target genes. It is very likely that Bmi-1 is negatively regulated by Max-Mad and Mnt-Max complexes. Thus, our studies suggest that c-Myc and other E-box binding proteins may positively or negatively regulate Bmi-1, which in turn regulates oncogenesis and stem cell-ness.

Our cell culture data showing an inverse correlation between Bmi-1 and Mel-18 expression prompted us to examine if indeed this inverse correlation exists in vivo in breast tumors. Bmi-1 is overexpressed in invasive breast cancer; we reasoned that in such breast tumors where Bmi-1 is highly expressed, Mel-18 expression might be low. Since the process of oncogenesis is multi factorial and heterogeneous, we further reasoned that in a minority of tumors, Bmi-1 expression may not be high; such tumors are expected to express normal to high Mel-18. Indeed, we found a strong negative correlation between Mel-18 and Bmi-1 expression in invasive breast cancer, which favors high Bmi-1 and low Mel-18 expression. Mel-18 and Bmi-1 expression also negatively correlated in normal tissues adjacent to tumors in four samples that we were able to analyze.

Although, our study used only a limited number of breast tumors, Bmi-1 is overexpressed in a variety of human cancers. Analysis of Mel-18 and Bmi-1 co-expression in a large cohort of breast tumors and other cancers remains to be explored. Correlation of Bmi-1 and Mel-18 with other clinico-pathological markers is also understudied. Nonetheless, our studies suggest that Mel-18 is physiological regulator of Bmi-1 expression in human fibroblasts and breast epithelial cells. The mechanisms of Mel-18 downregulation in tumors also remain to be elucidated. Based on these results, we suspect

that this inverse correlation may persist with other cell types and cancers of various organs. In particular, it has been suggested that Bmi-1 may be a cancer stem cell marker (14, 28); it will be interesting to explore whether Mel-18 downregulation in certain specific cell types makes them susceptible to cancer stem cell conversion.

In summary, our studies suggest that the Mel-18-c-Myc-Bmi-1-p16-pRb pathway could be targeted for cancer therapy. Although there has already been considerable interest in c-Myc, p16 and pRb, our data suggest that PcG protein Mel-18 and Bmi-1 are also valid targets for cancer therapy. For example, restoration of Mel-18 expression or ablation of Bmi-1 expression in tumors by various therapeutic approaches might help in cancer treatment. Lastly, as stem cell defect has been linked to various age-related pathologies (18, 39), we speculate that Mel-18 may play an important role in the development of age-related ailments by virtue of downregulating Bmi-1, a known regulator of stem cell-ness.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Mel-18 downregulates Bmi-1 in human cells:

A. Overexpression of Mel-18 but not other related PcG proteins downregulate Bmi-1 expression; MCF10A cells were infected with retrovirus vectors expressing the indicated PcG protein, selected and expanded. Total protein lysate was analyzed by western blot analysis to determine the expression of Bmi-1, β-actin (loading control) and PcG protein Mel-18, MBLR and NSPC1 as described in experimental procedures. PcG proteins, which contain Myc tag were detected by c-Myc antibody.

B. Stable expression of Mel-18 in MCF10A, MCF7 and MRC-5 fibroblasts leads to downregulation of Bmi-1 and c-Myc oncoprotein. Total cell lyaste from indicated cells was analyzed by western blot analysis using antibody against Bmi-1, Mel-18, c-Myc and α-Tubulin (loading control), as described in experimental procedures.

C. Transient overexpression of Mel-18 in 293T cells leads to the downregulation of c-Myc and Bmi-1 in a dose-dependent manner. 293 T cells were transiently transfected with increasing amount pLPC-Mel-18, and 48 hrs post-transfection, the total cell lysate was analyzed by western blot analysis using antibody against Mel-18, Bmi-1 and GFP (transfection control).

D. and E. Stable expression of Mel-18 RNAi in MCF10A and MRC-5 cells leads to upregulation of c-Myc and Bmi-1 expression. MCF10A (D) or MRC-5 (E) cells were infected with pRS vector expressing either Mel-18 shRNA #1 (#1i), Mel-18 shRNA #2 (#2i) or an irrelevant control shRNA (Ctrl i), drug selected and analyzed

for the expression of Mel-18, c-Myc and Bmi-1 as described above and in experimental procedures.

Figure 2. Structural analysis of Mel-18:

A. Schematic representation of mutants of Mel-18 depicting various domains.

B. Stable overexpression of wild type (WT) and the mutants of Mel-18 in MCF10A cells; WT and PS mutant downregulated Bmi-1 expression, whereas overexpression of ΔRF and ΔRFNLS mutants led to upregulation of Bmi-1. WT or mutants of Mel-18 were stably expressed using retroviral expression as described in experimental procedures. Bmi-1, Mel-18 and α-tubulin were detected as described in Fig. 1.

C. Transient overexpression of WT and various mutants of Mel-18 in 293T cells; Similar to stable overexpression, WT and PS mutants caused downregulation of Bmi-1, whereas overexpression of ΔRF and ΔRFNLS mutants upregulated Bmi-1 in 293T cells. 293T cells were transiently trasnfected with 0, 1 and 3 μg of Mel-18 expressing vector and analyzed for Bmi-1, Mel-18 and GPF (transfection control) expression as described in Fig. 1.

Figure 3. Mel-18 regulates Bmi-1 at the transcription level:

A. Treatment with MG132, a proteosome inhibitor does not restore Bmi-1 expression in Mel-18 overexpressing cells. Control, Mel-18 and Bmi-1 overexpressing cells were treated with 10 μm MG132 for the indicted time period, and analyzed by western blot analysis for the expression of Bmi-1, Mel-18 and α-tubulin as described in Fig. 1.

B. Bmi-1 half-life is similar in control and Mel-18 overexpressing cells. Vector control and Mel-18 overtexpressing cells were treated with 100 µg/ml cyclohexamide (CHX) for the indicated amounts of time and analyzed for the expression of Bmi-1, Mel-18 and β-actin. The percent remaining Bmi-1 protein was calculated by densitometry of the Bmi-1 signal present in different lanes and normalizing it with the β-actin control signal present in the corresponding lanes.

C. RT PCR analysis of Bmi-1 in Mel-18 overexpressing and control cells. Different quantities (as indicated) of total RNA were reverse transcribed into cDNA, and amplified using Bmi-1- and β-actin- specific primers as described in experimental procedures.

Figure 4. Mel-18 regulates Bmi-1 via c-Myc repression:

A. Bmi-1 promoter contains a c-Myc binding site (E-Box), (indicated in bold and red color), and several Sp-1 binding sites and GC boxes (shown in bold and green color). Bmi-1 promoter sequence was obtained from the Gene Bank and analyzed for the presence of transcription factor binding sites as described in experimental procedures.

B. c-Myc binds to the E Box sequences in the Bmi-1 promoter as shown by the ChIP analysis. ChIP analysis was performed using vector control or Mel-18 overexpressing cell lysates IPEd using c-Myc antibody or control IgG, and a primer set that either amplifies the c-Myc binding flanking region in Bmi-1 promoter (c-Myc site) or a region further upstream, which does not contains c-Myc binding site (Non-Myc site).

C. c-Myc binding region of Bmi-1 promoter imparts c-Myc regulated promoter activity to a minimal promoter. The c-Myc binding region of Bmi-1 promoter was PCR amplified and cloned upstream of a minimal promoter present in the pluc vector to construct pLuc-Myc. The luciferase reporter assays after transient transfection of pLuc and pLuc-Myc together with different amounts (as indicated) of c-Myc expressing plasmid were carried out as described in experimental procedures.

D. Detailed analysis of Bmi-1 promoter activity. The pGL-Bmi PrWT, pGL-Bmi PrMut and pGL-Bmi PrΔMyc reporters (described in the text) were analyzed for the luciferase activity in 293T cells as described in experimental procedures.

E. F and G. Overexpression of Mel-18 downregulated, while c-Myc overexpression upregulated Bmi-1 promoter activity through c-Myc binding site (E and F respectively). Similarly c-Myc knockdown using transient transfection of a plasmid containing c-Myc shRNA downregulated activity of Bmi-1 promoter, which contains intact c-Myc binding site (G). The promoter activity was analyzed 48 hr after transfecting 293T cells with promoter-reporter constructs and different amounts of Mel-18, c-Myc or c-Myc shRNA expressing plasmids as indicated.

Figure 5. c-Myc regulates Bmi-1 expression:

A. Stable overexpression of c-Myc leads to Bmi-1 upregulation. MCF10A cells were infected with a c-Myc expressing retrovirus (pLNCX2-Myc), drug selected and the expression of c-Myc, Bmi-1 and α-tubulin was determined by western blot analysis as described in Fig. 1.

B. Knockdown of c-Myc expression by RNAi approach leads to downregulation of endogenous Bmi-1. MCF10A cells expressing c-Myc shRNAs (Myc RNAi #1 and Myc RNAi #2) were generated and analyzed for the expression of c-Myc, Bmi-1 and α-tubulin by western blot analysis as described in Fig. 1.

C. Restoration of c-Myc in Mel-18 overexpressing cells by its transient overexpression leads to reversal of Bmi-1 repression by Mel-18. 293T cells were transfected with either Mel-18, c-Myc or both and a GFP expressing plasmid. The total cell lysate from each set was analyzed for the expression of Mel-18, c-Myc, Bmi-1 and GFP by western blot analysis as described in Fig. 1.

Figure 6. Mel-18 is overexpressed in senescent fibroblasts and the forced expression of Mel-18 accelerates cellular senescence in proliferating fibroblasts:

A. Overexpression of Mel-18 and p16, and downregulation of Bmi-1 during senescence in fibroblasts. MRC-5, BJ and WI-38 fibroblasts were serially passaged in culture until senescence as determined by measuring the SA-β-gal index. Mel-18, Bmi-1, pRb, p16 and α-tubulin in total cell lysates from proliferating presenescent (Y) and senescent (O) cultures were detected by western blot analysis as described in experimental procedures.

B. Mel-18 is not upregulated during quiescence in WI-38 fibroblasts. Proliferating presenescent (YGr) cells were made quiescent by serum deprivation for 72 hr. Mel-18, Bmi-1, pRb and p16 expression in proliferating presenescent (YGr), presenescent quiescent (YQ) and senescent cells was determined by western blot analysis as described in experimental procedures.

C. Mel-18 overexpression leads to accelerated senescence in MRC-5 fibroblasts. MRC-5 fibroblasts expressing Mel-18 or Bmi-1, and vector-infected control cells were serially passaged in culture to determine the replicative life span as described (Itahana et al. 2003). SA-β-gal index was determined as described (Dimri et al. 1995).

D. Similar to Mel-18 overexpression, knockdown of Bmi-1 expression leads to accelerated senescence in MRC-5 fibroblasts. MRC-5 cells expressing a control shRNA (Ctrl), or Bmi-1 shRNAs (Bmi-1 RNAi#1 and Bmi-1 RNAi #2 were serially passaged in culture to determine the replicative life span as described in Fig. 6C. SA-β-gal index was determined as described (Dimri et al. 1995).

E. Knockdown of Mel-18 expression leads to the extension of replicative life span in MRC-5 fibroblasts. Mel-18 shRNAs (Mel-18 RNAi #1, Mel-18 RNA #2) expressing and control cells were passaged in culture to determine replicative life span and onset of senescence as described (Itahana et al. 2003, Dimri, et al. 1995).

F. Mel-18 overexpression and knockdown of Bmi-1expression accelerates senescence via p16-pRb pathway; Western blot analysis of p16, pRb and p53 in control, and Mel-18 and Bmi-1 overexpressing cells (left panel), and control and Bmi-1 knockdown cells (right panel) was performed as described in Fig. 6A.

Figure 7A. Reduction of transformed phenotype by Mel-18 overexpression and knockdown of Bmi-1 expression: Overexpression of Mel-18 and knockdown of Bmi-1 expression in MCF7 decreases colony formation in soft-agar. Control or Mel-18 overexpressing MCF7 cells (upper panel), and control (Ctrl i) or Bmi-1 shRNAs (Bmi-1 i

#1 and Bmi-1 i #2) cells (lower panel) were plated in soft-agar to determine the anchorage independent growth as described in materials and methods. Colonies from three different experiments were counted and plotted, Western blot analysis of Bmi-1 and Mel-18 was performed to measure the efficacy of Bmi-1 knockdown and Mel-18 overexpression.

Figure 7B. Mel-18 and Bmi-1 expression inversely correlates in breast tumors: A representative figure of two tumor samples; sample 1 expresses high Bmi-1 and low Mel-18, while the second sample expresses high Mel-18 and low Bmi-1 expression. Tumor adjacent normal tissue of a biopsy sample with high Mel-18 and low Bmi-1 is also shown (lower panel). Tissues were stained with Bmi-1- or Mel-18- specific antibodies and counterstained with hematoxylin as described in experimental procedures.

Figure 1 **Guo et al.**

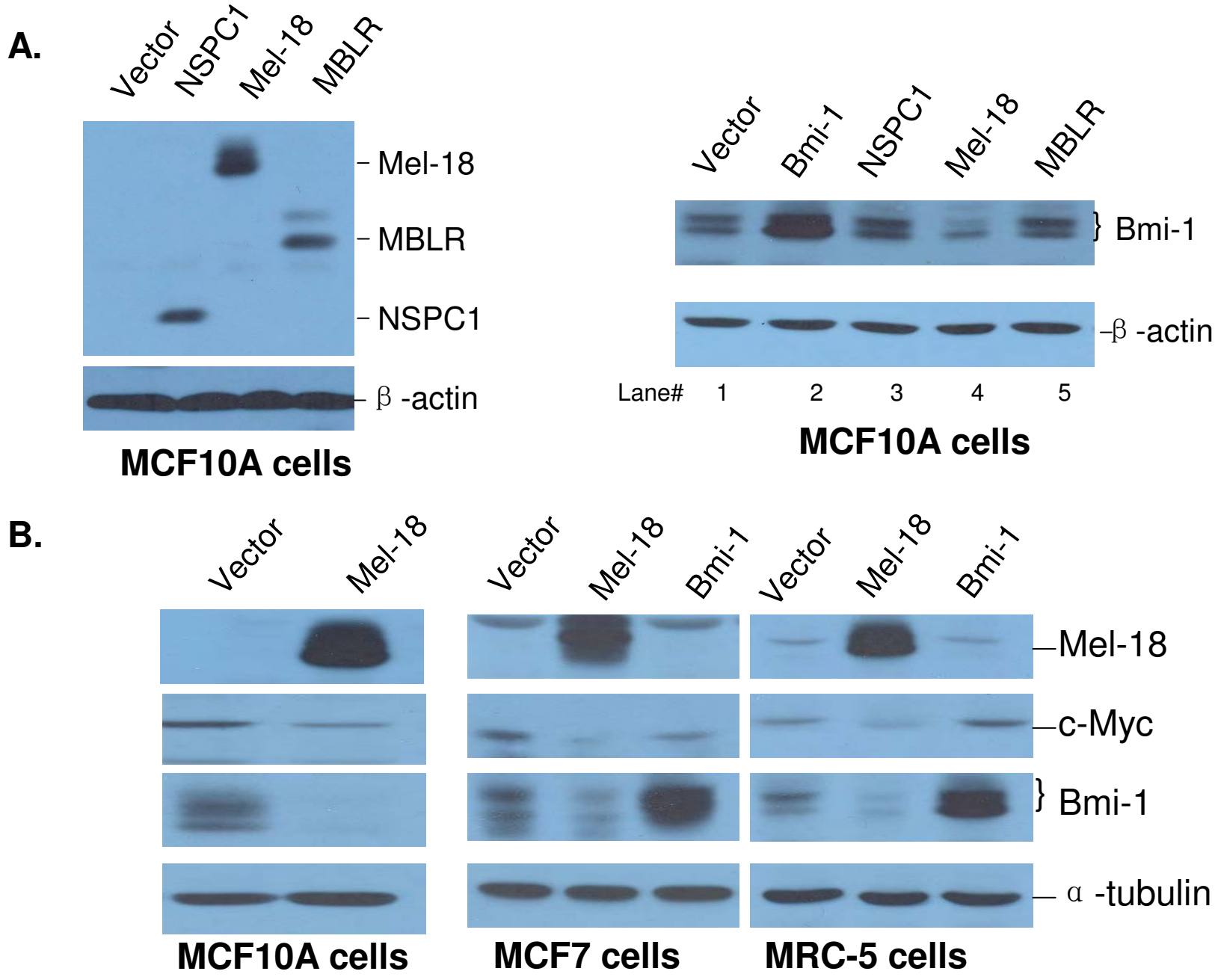


Figure 1

Guo et al.

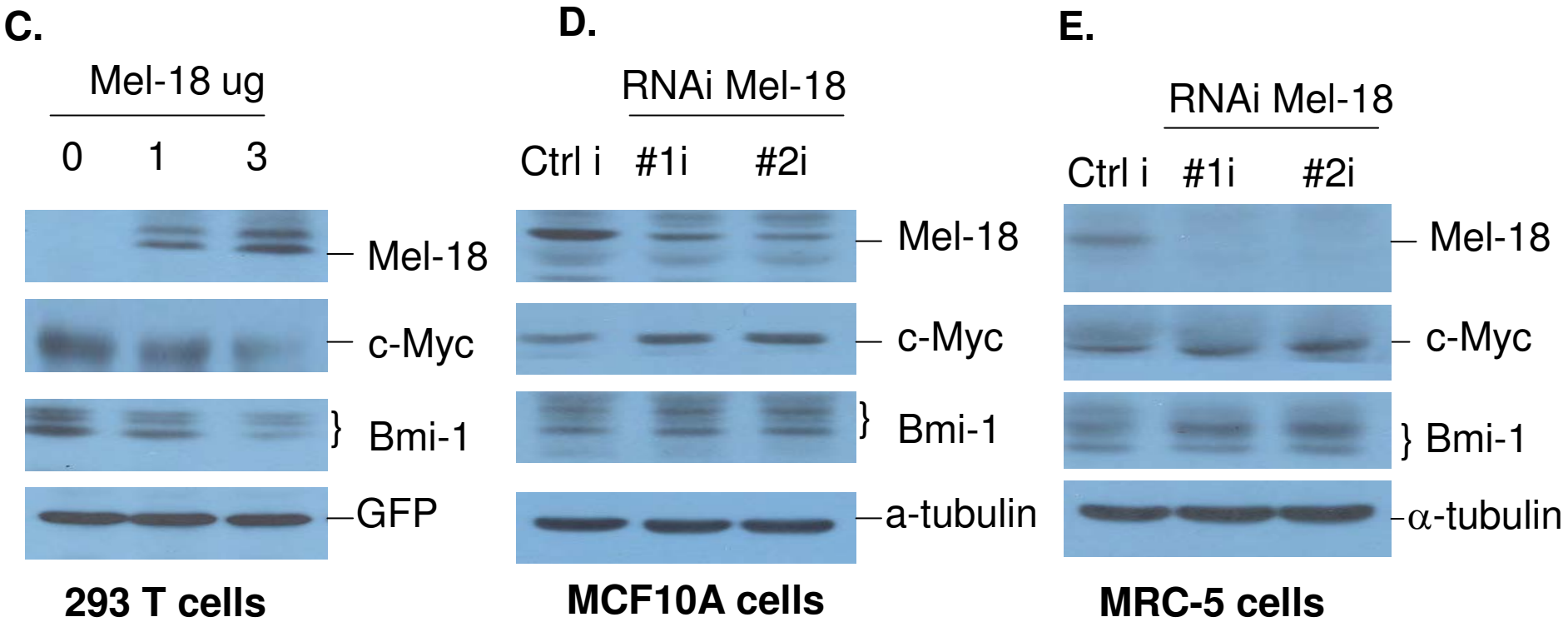
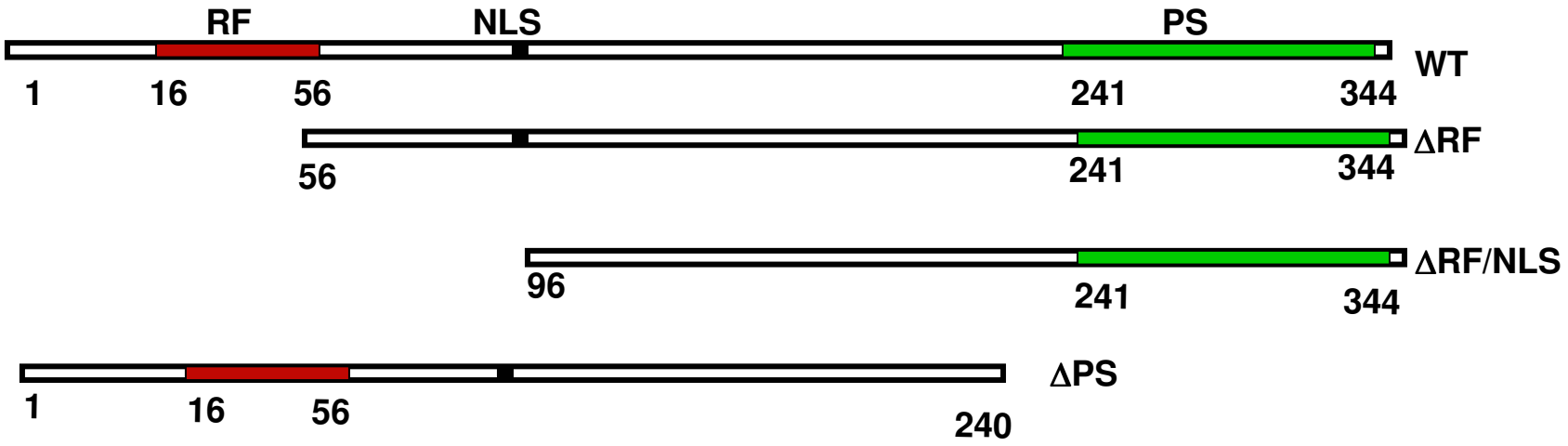


Figure 2 **Guo et al.**

A.



B.

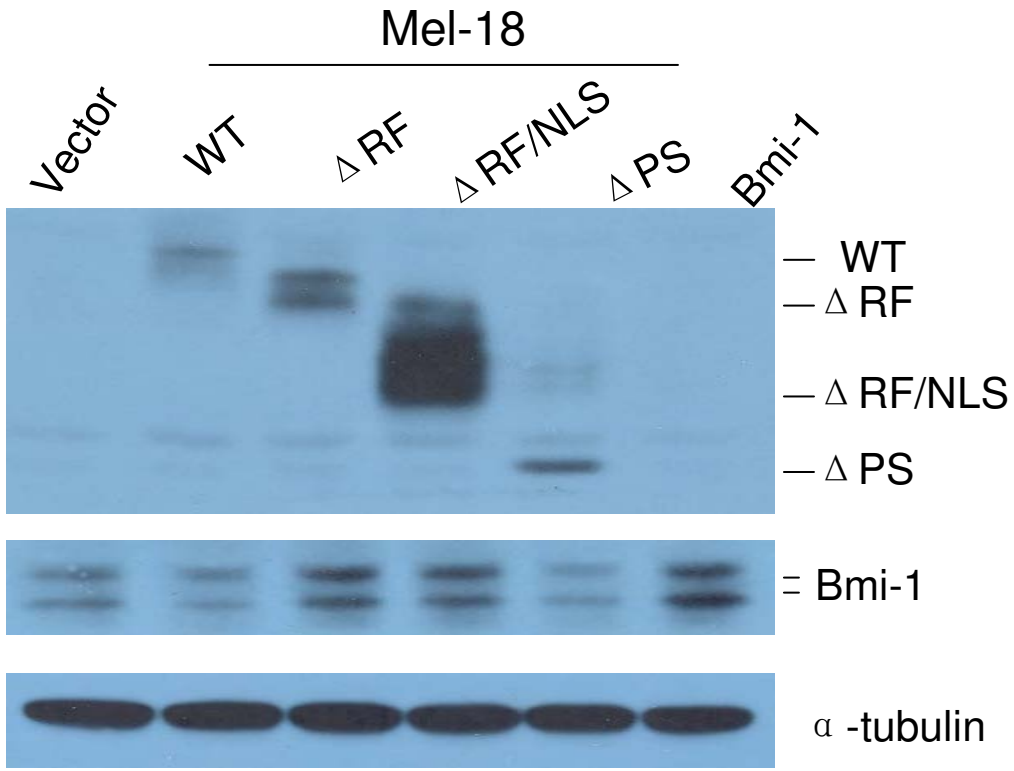


Figure 2 **Guo et al.**

C.

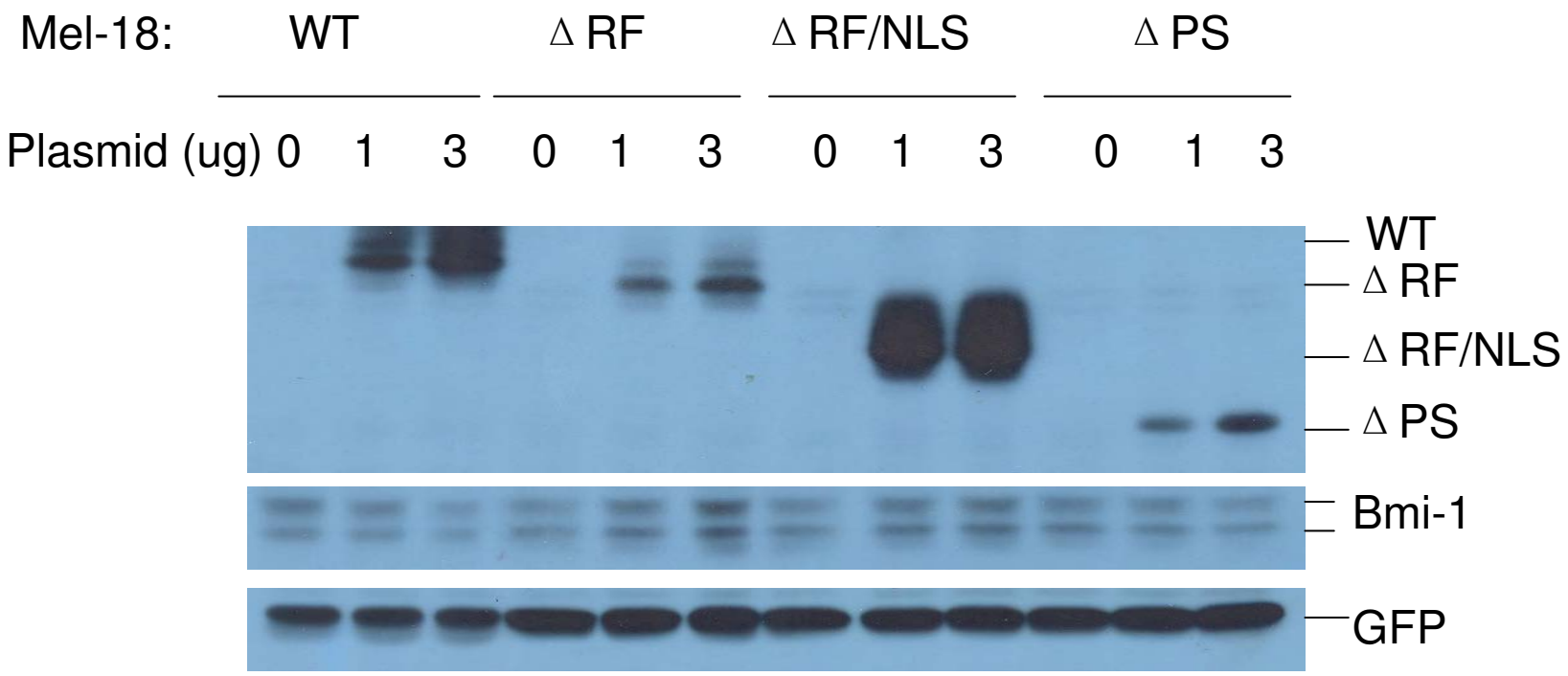


Figure 3 **Guo et al.**

A.

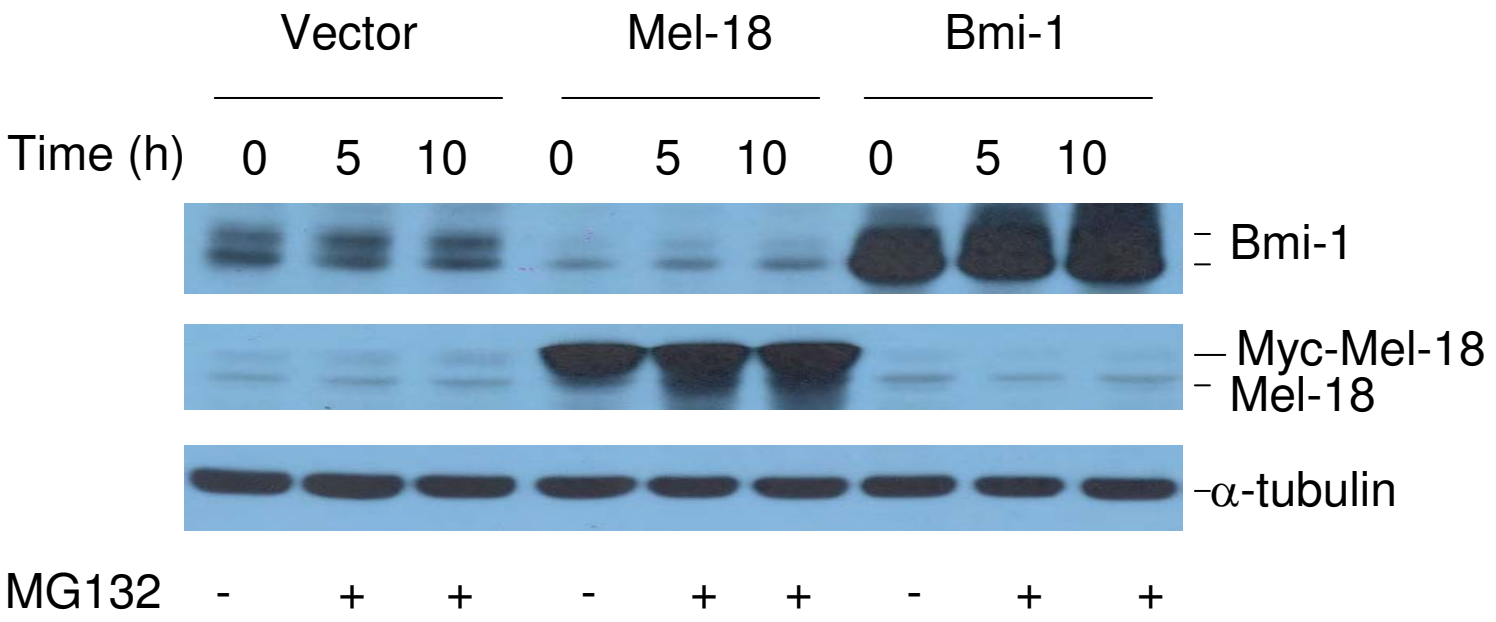
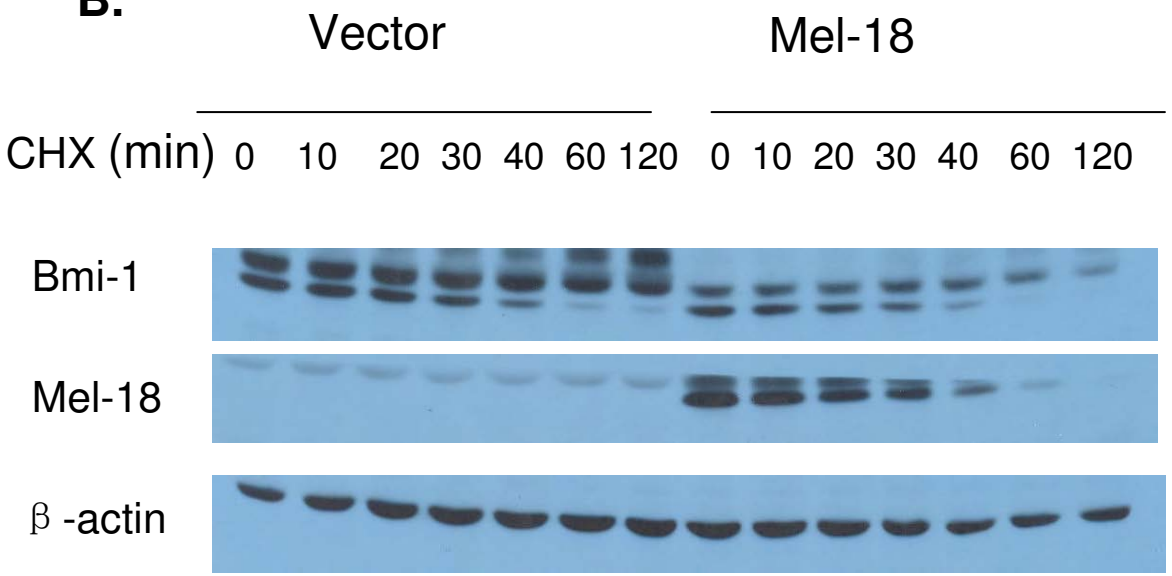
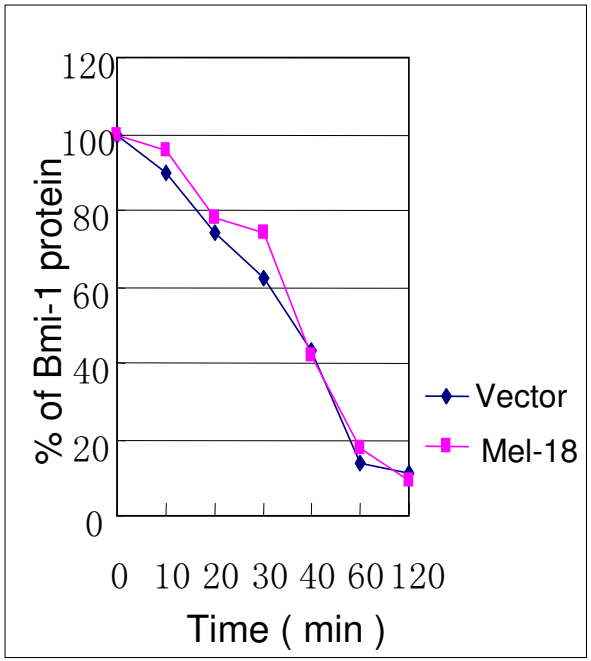


Figure 3 **Guo et al.**

B.





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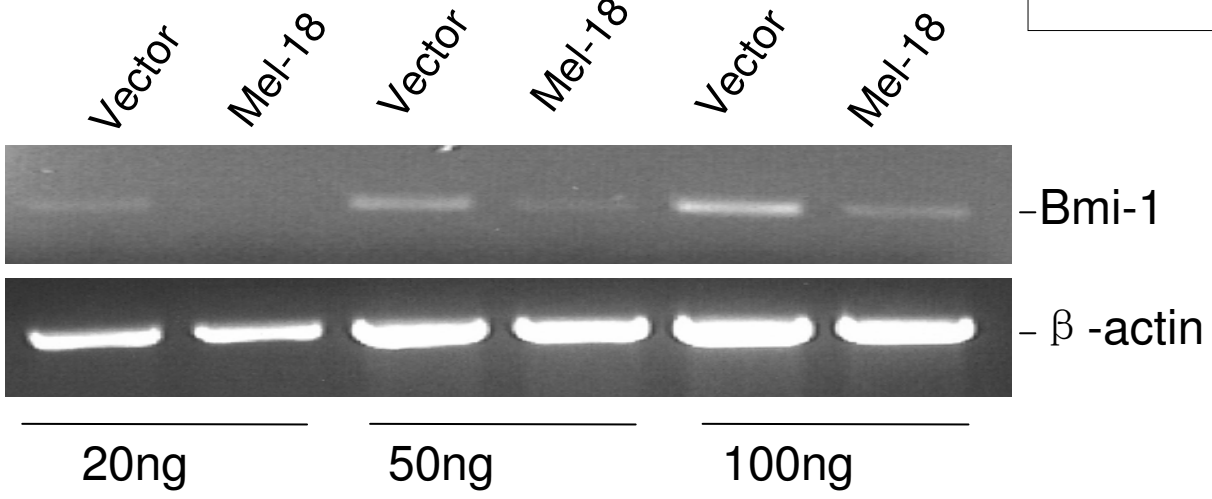

Figure 4. **Guo et al.**

A.

-400

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CCCGCTG**GGGGGGCGGCGGGGGG**AG**GGGGCGGG**CGGACTCCGAGCCATTTTGGAGCCGGTGTCAGTTTCCACTCTG
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B.

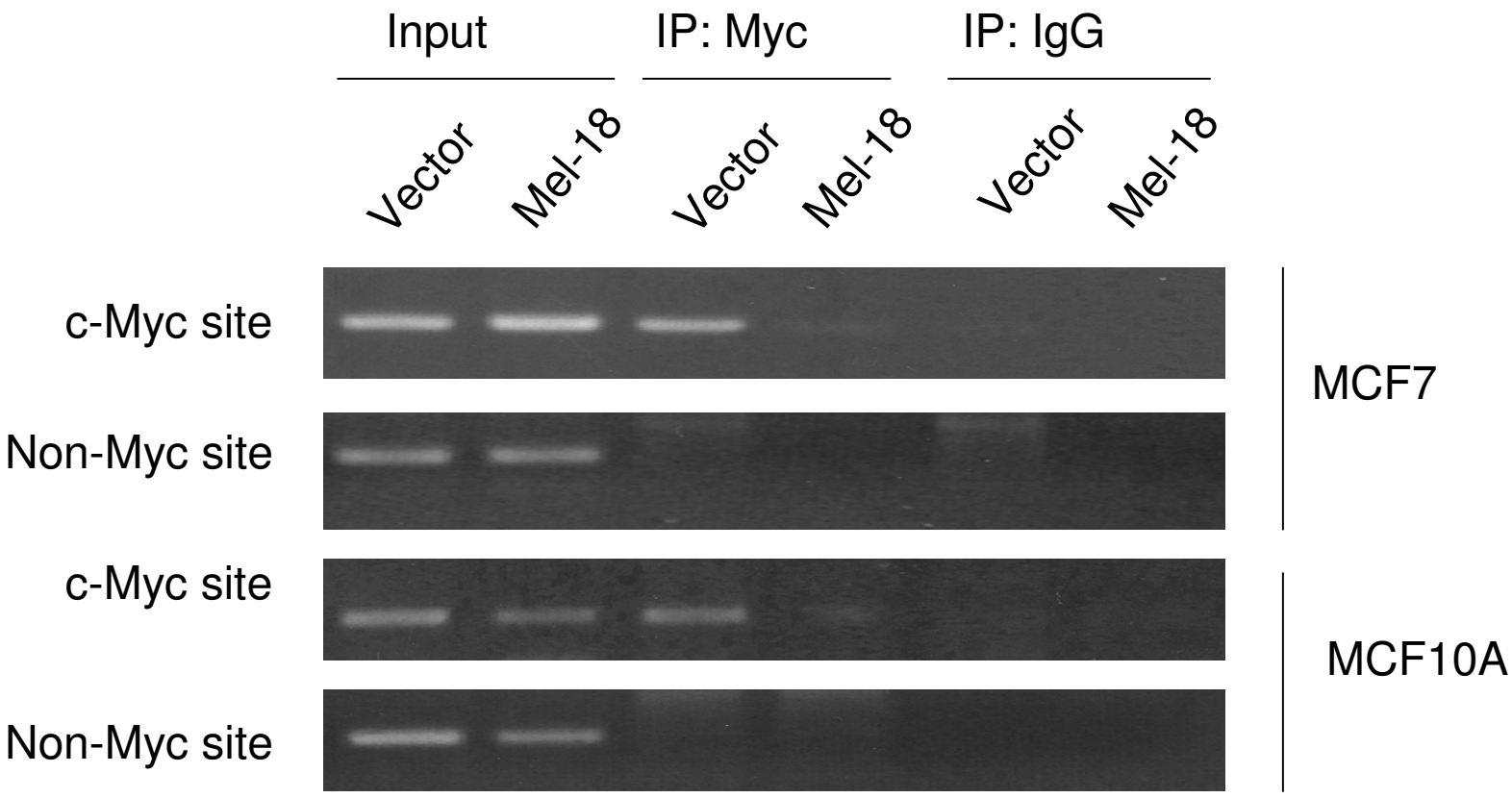
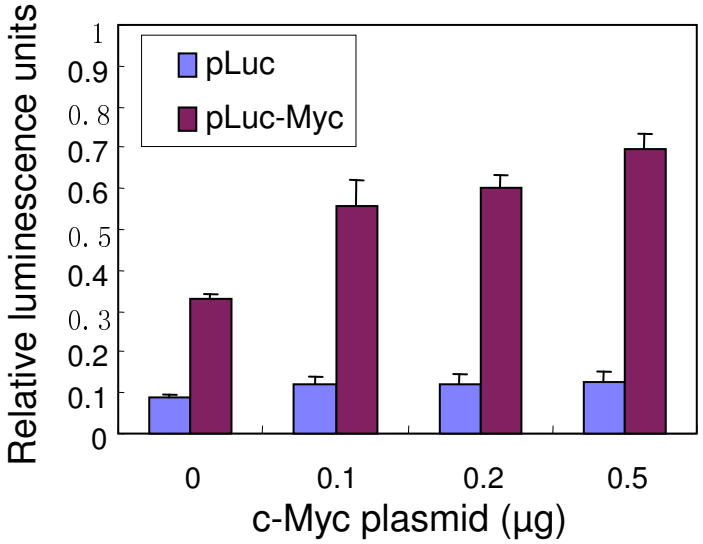
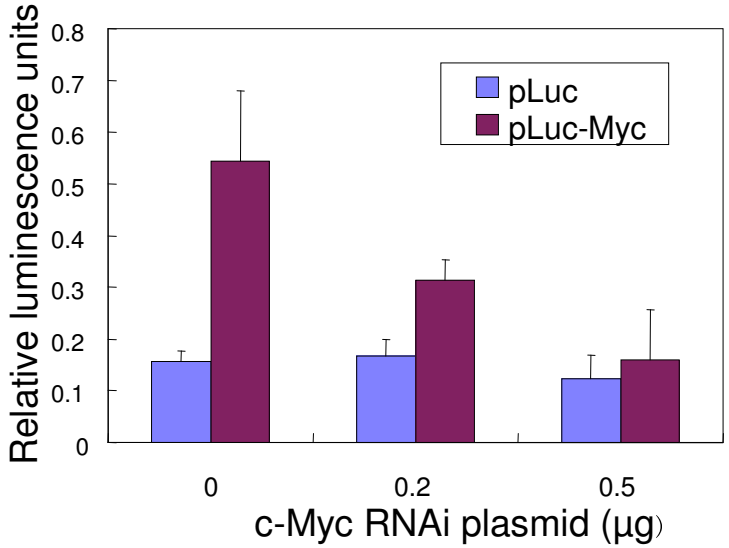


Figure 4 **Guo et al.**

C.





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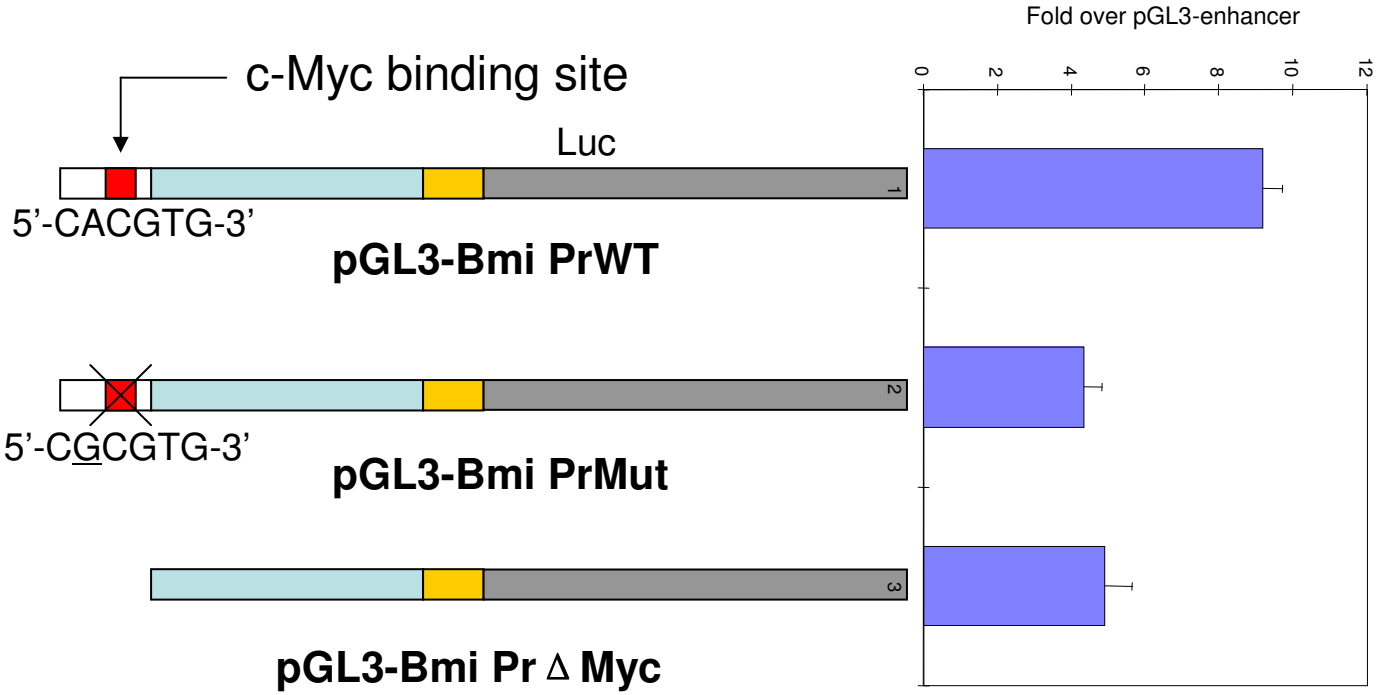
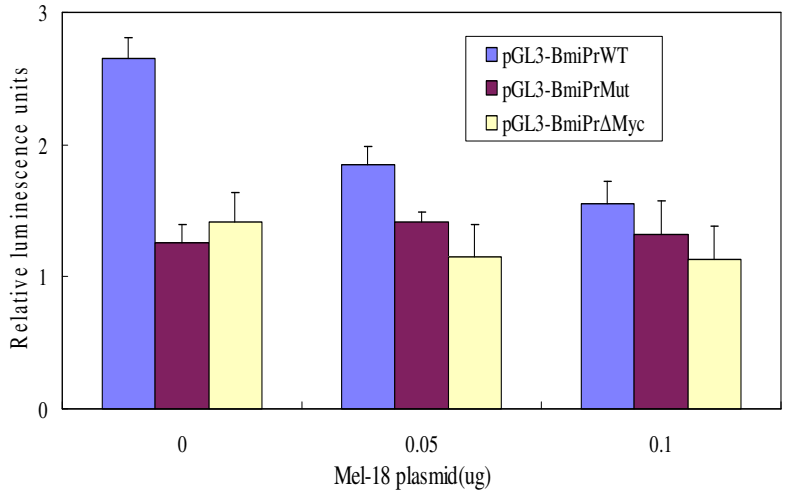


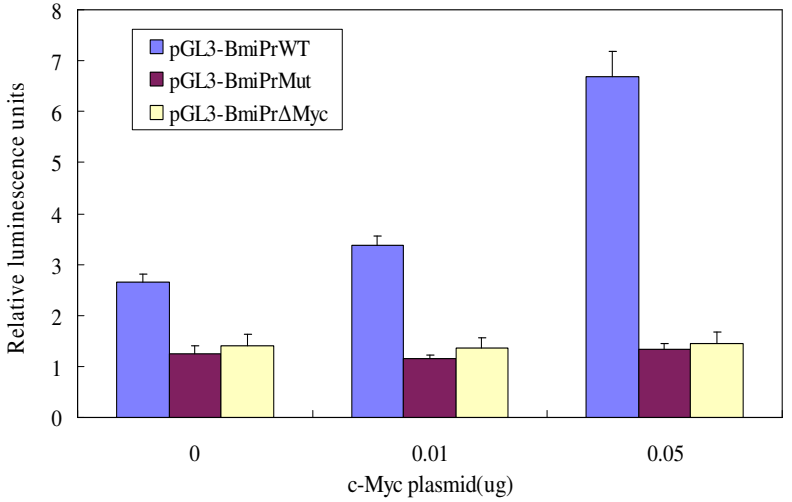
Figure 4

Guo et al.

E.



F.



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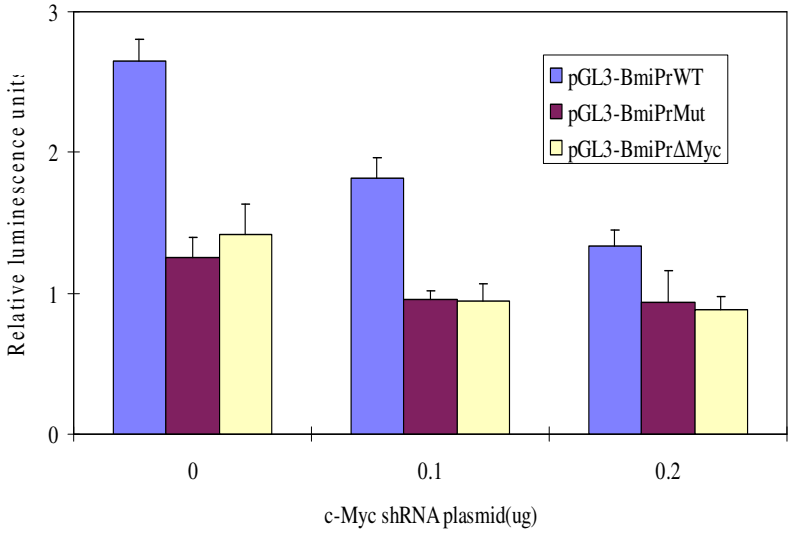


Figure 5

Guo et al.

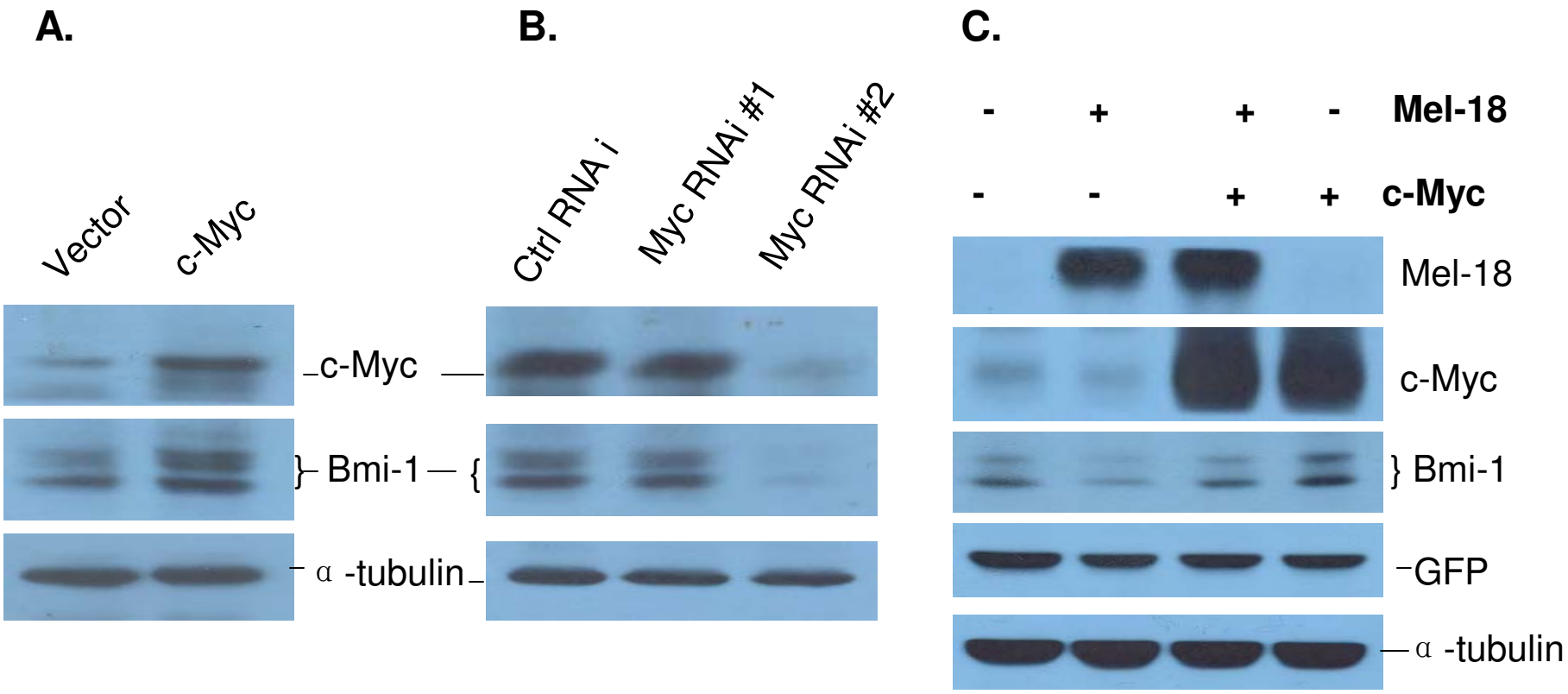


Figure 6

Guo et al.

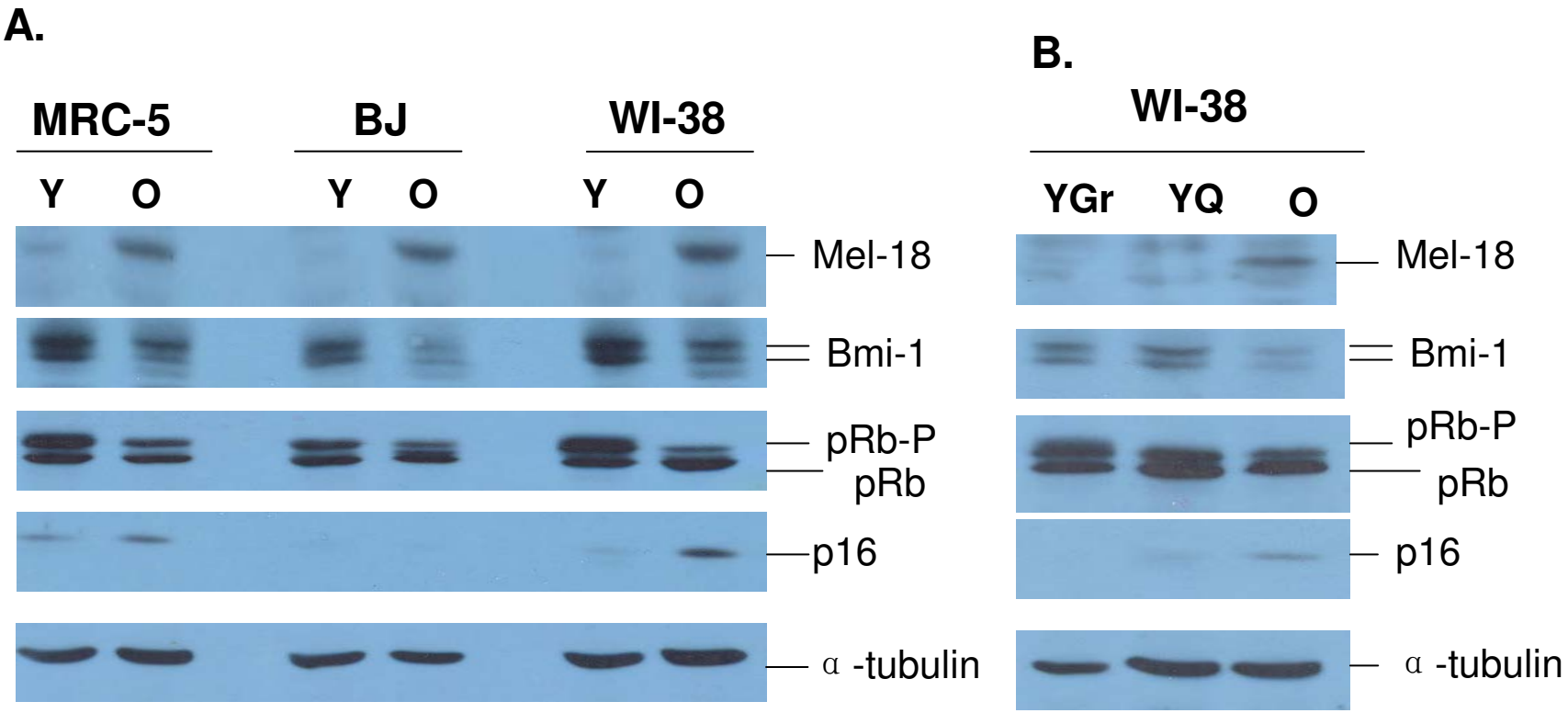
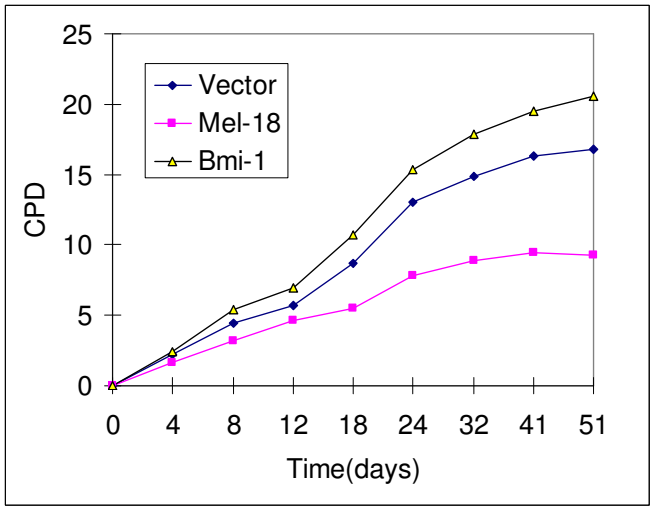
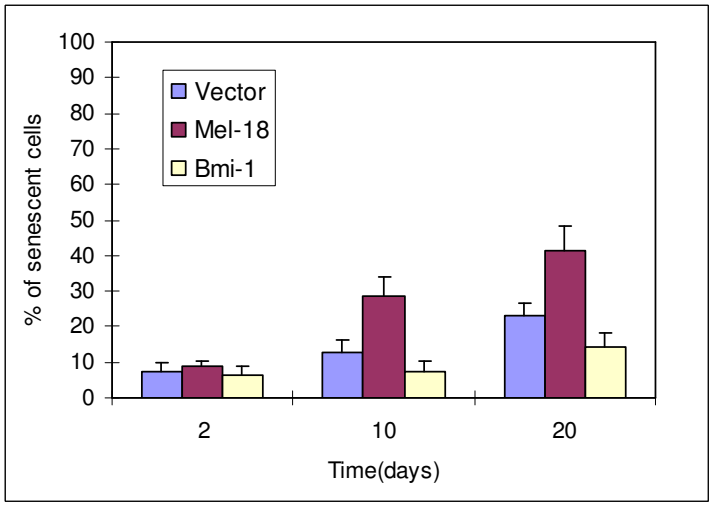


Figure 6 **Guo et al.**

C.





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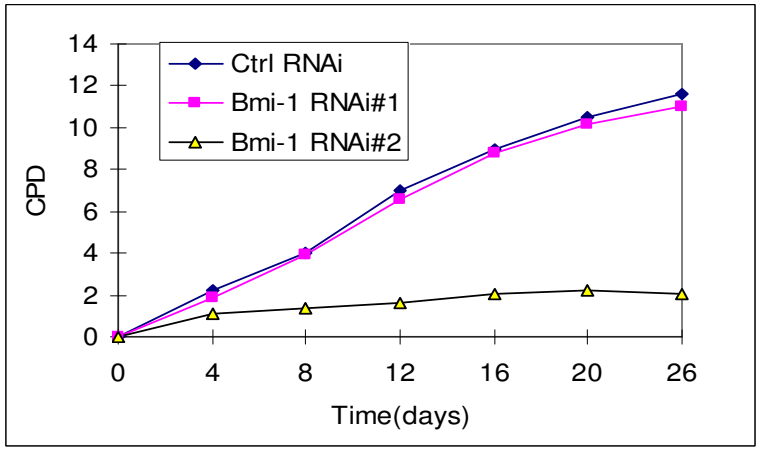
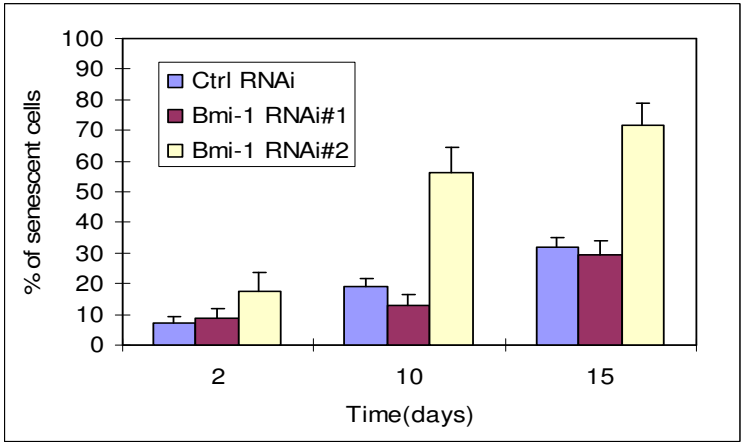
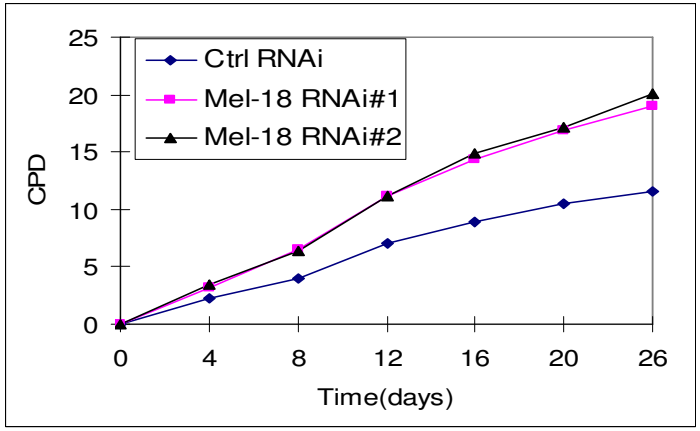




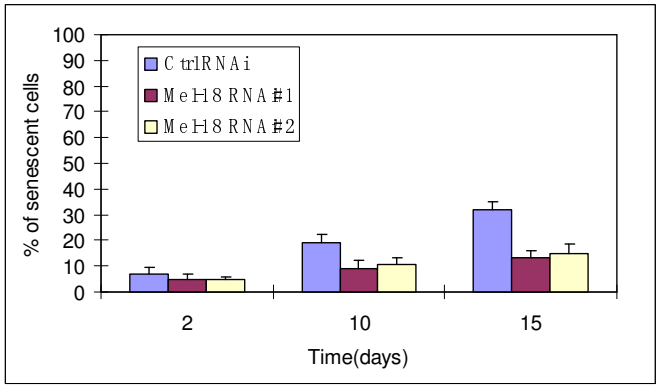
Cell growth curve **SA- β -gal Assay**

Figure 6 **Guo et al.**

E.



Cell growth curve



SA- β -gal Assay

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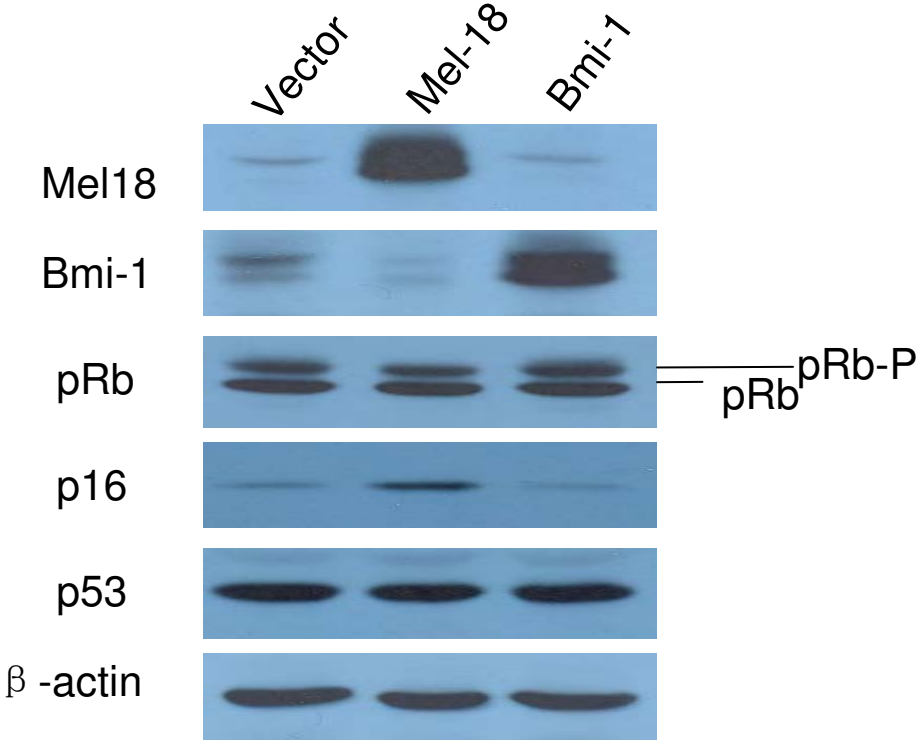


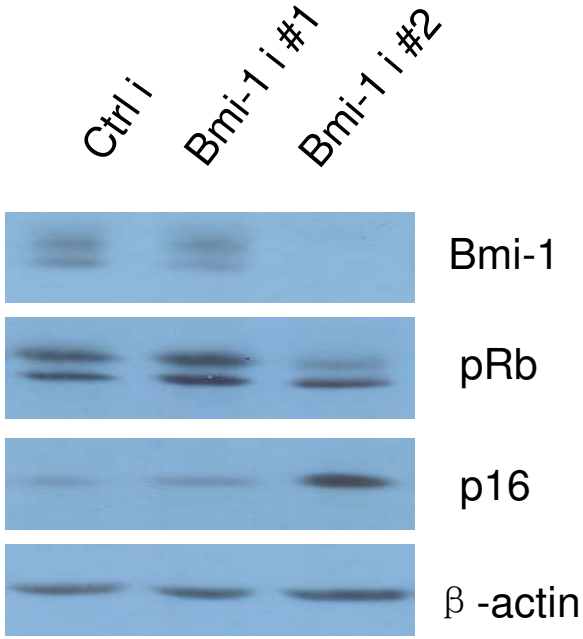
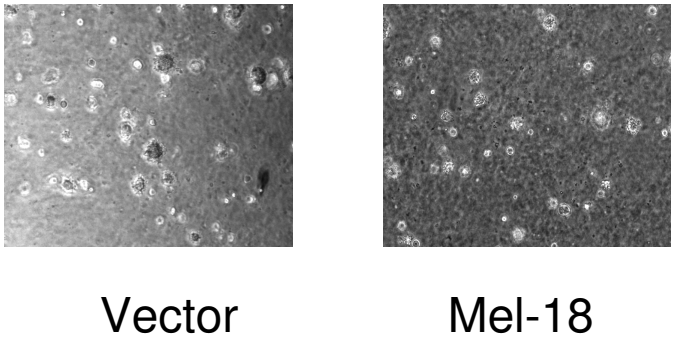
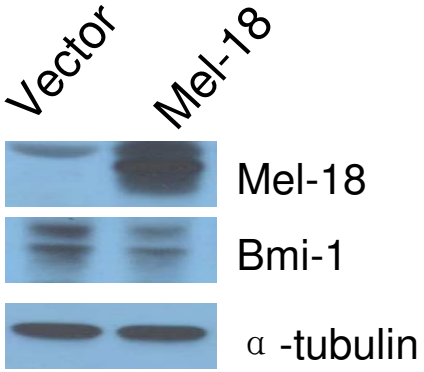
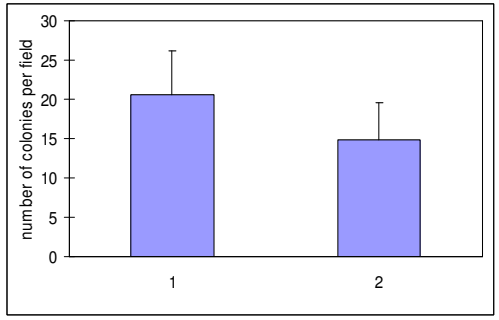


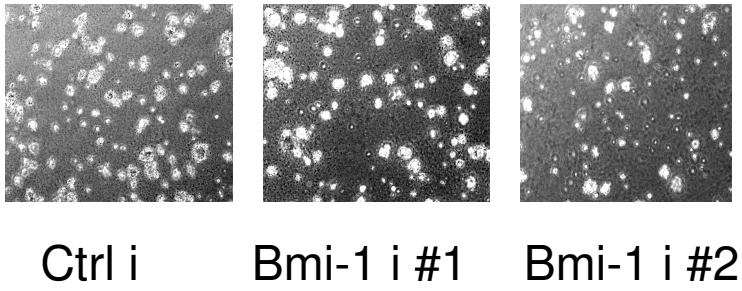
Figure 7A

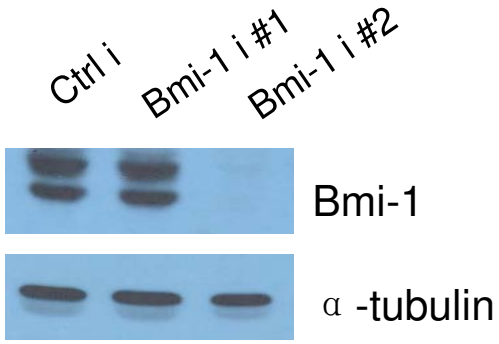
Guo et al.











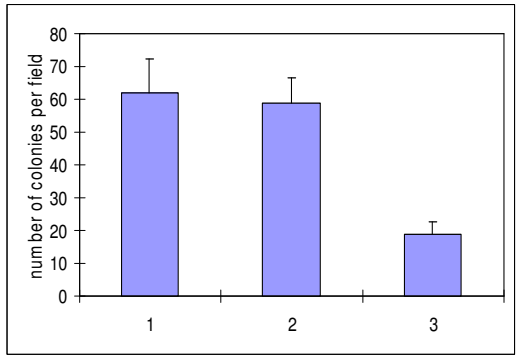
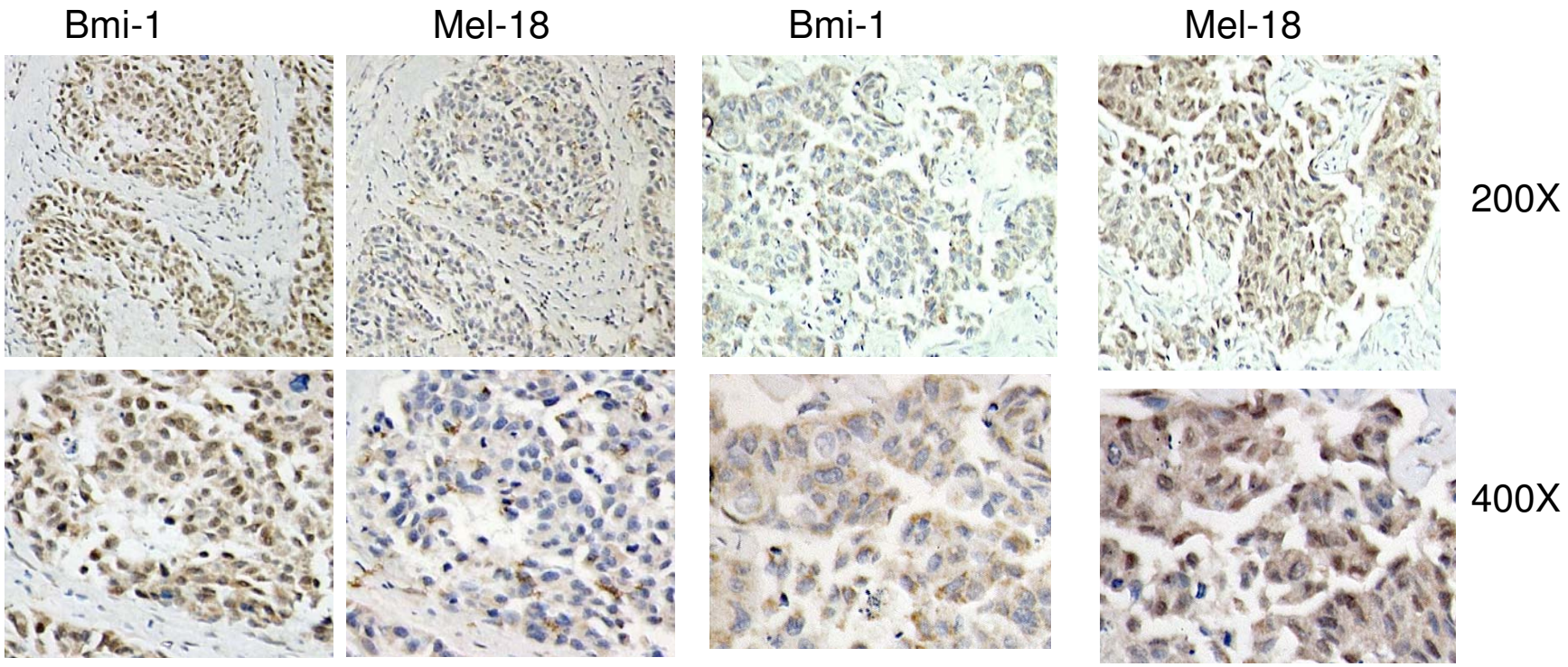
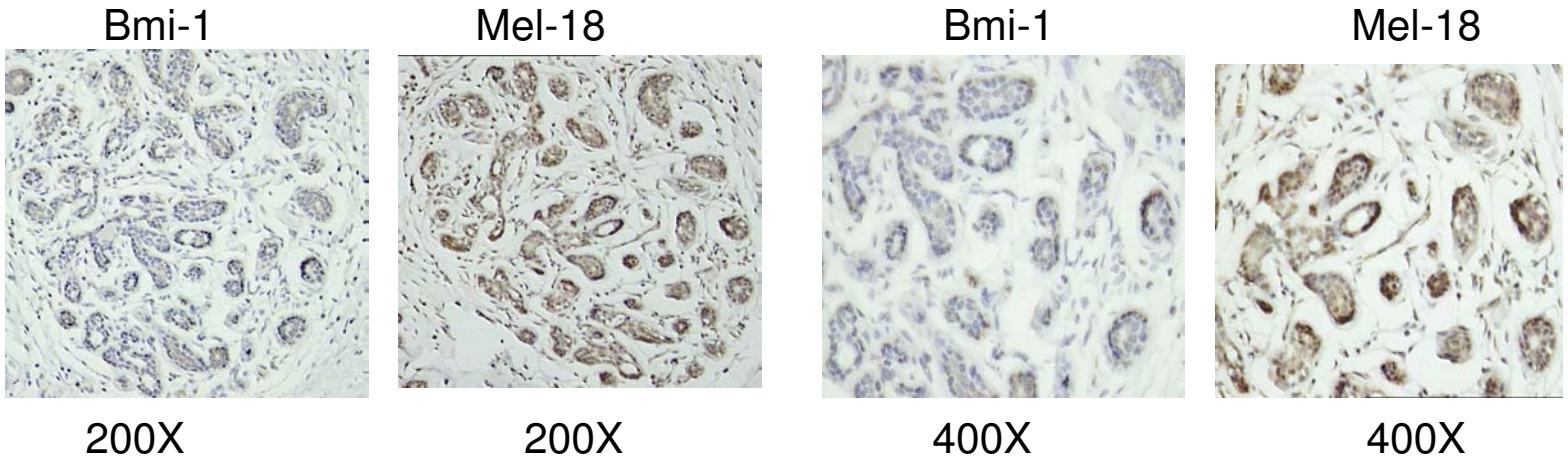


Figure 7B

Guo et al.

Tumor Tissues





Tumor Adjacent Normal Tissue